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(54) Title: HUMAN ANTIBODIES DERIVED FROM IMMUNIZED XENOMICE

(57) Abstract

Antibodies with fully human variable regions against a specific antigen can be prepared by administering the antigen to a transgenic animal which has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled. Various subsequent manipulations can be performed to obtain either antibodies *per se* or analogs thereof.

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HUMAN ANTIBODIES DERIVED FROM IMMUNIZED XENOMICE

Technical Field

The invention relates to the field of immunology, and in particular to the production of antibodies. More specifically, it concerns producing such antibodies by a process which includes the step of immunizing a transgenic animal with an antigen to which antibodies are desired. The transgenic animal has been modified so as to produce human, as opposed to endogenous antibodies.

Background Art

PCT application WO 94/02602, published 3 February 1994 and incorporated herein by reference, describes in detail the production of transgenic nonhuman animals which are modified so as to produce antibodies with fully human variable regions rather than endogenous antibodies in response to antigenic challenge. Briefly, the endogenous loci encoding the light and heavy immunoglobulin chains are incapacitated in the transgenic hosts and loci encoding human heavy and light chain proteins are inserted into the genome. In general, the animal which provides all the desired modifications is obtained by cross-breeding intermediate animals containing fewer than the full complement of modifications. The preferred embodiment of the nonhuman animal described in the specification is a mouse. Thus, mice, specifically, are described which, when administered immunogens, produce antibodies with human variable regions, including fully human antibodies, rather than murine antibodies that are immunospecific for these antigens.

The availability of such transgenic animals makes possible new approaches to the production of fully human antibodies. Antibodies with various immunospecificities are desirable for therapeutic and diagnostic use. Those antibodies intended for human therapeutic and *in vivo* diagnostic use, in particular, have been problematic because prior art sources for such antibodies resulted in immunoglobulins bearing the characteristic structures of antibodies produced by nonhuman

hosts. Such antibodies tend to be immunogenic when used in humans.

The availability of the nonhuman, immunogen-responsive transgenic animals described in the above-referenced WO 94/02602 make possible convenient production of human antibodies without the necessity of employing human hosts.

Disclosure of the Invention

The invention is directed to methods to produce human antibodies by a process wherein at least one step of the process includes immunizing a transgenic nonhuman animal with the desired antigen. The modified animal fails to produce endogenous antibodies, but instead produces B-cells which secrete immunoglobulins with fully human variable regions. The antibodies produced include fully human antibodies and can be obtained from the animal directly, or from immortalized B-cells derived from the animal. Alternatively, the genes encoding the immunoglobulins with human variable regions can be recovered and expressed to obtain the antibodies directly or modified to obtain analogs of antibodies such as, for example, single chain F_V molecules.

Thus, in one aspect, the invention is directed to a method to produce an immunoglobulin with a fully human variable region to a specific antigen or to produce an analog of said immunoglobulin by a process which comprises immunizing a nonhuman animal with the antigen under conditions that stimulate an immune response. Fully human immunoglobulins are included in this group and are preferred. The nonhuman animal is characterized by being substantially incapable of producing endogenous heavy or light immunoglobulin chain, but capable of producing immunoglobulins either with both human variable regions and constant regions or with fully human variable regions or both. In the resulting immune response, the animal produces B cells which secrete immunoglobulins, with at least variable regions that are fully human, specific for the antigen. The human immunoglobulin of desired specificity can be directly recovered from the animal, for example, from the serum, or primary B cells can be obtained from the animal and

immortalized. The immortalized B cells can be used directly as the source of human antibodies or, alternatively, the genes encoding the antibodies can be prepared from the immortalized B cells or from primary B cells of the blood or lymphoid tissue (spleen, tonsils, lymph nodes, bone marrow) of the immunized animal and expressed in recombinant hosts, with or without modification, to produce the immunoglobulin or its analogs. In addition, the genes encoding the repertoire of immunoglobulins produced by the immunized animal can be used to generate a library of immunoglobulins to permit screening for those variable regions which provide the desired affinity. Clones from the library which have the desired characteristics can then be used as a source of nucleotide sequences encoding the desired variable regions for further manipulation to generate antibodies or analogs with these characteristics using standard recombinant techniques.

In another aspect, the invention relates to an immortalized nonhuman B cell line derived from the above described animal. In still another aspect, the invention is directed to a recombinant host cell which is modified to contain the gene encoding either the human immunoglobulin with the desired specificity, or an analog thereof which exhibits the same specificity.

In still other aspects, the invention is directed to antibodies or antibody analogs prepared by the above described methods and to recombinant materials for their production.

In still other aspects, the invention is directed to antibodies with fully human variable regions, including fully human antibodies which are immunospecific with respect to particular antigens set forth herein and to analogs which are similarly immunospecific, as well as to the recombinant materials useful in the production of these antibodies.

Brief Description of the Drawings

Figure 1 shows the serum titers of anti-IL-6 antibodies from a Xenomouse™ immunized with human IL-6 and which antibodies contain human κ light chains and/or human μ heavy chains.

Figure 2 shows the serum titers of anti-IL-8 antibodies from a Xenomouse™ immunized with human IL-8 and which antibodies contain human κ light chains and/or human μ heavy chains.

Figure 3 shows the serum titers of anti-TNFα antibodies from a Xenomouse™ immunized with human TNF-α and which antibodies contain human κ light chains and/or human μ heavy chains.

Figure 4 shows the serum titers of anti-CD4 antibodies from a Xenomouse™ immunized with human CD4 and which antibodies contain human κ light chains and/or human μ heavy chains.

Figure 5 shows the serum titers of a Xenomouse™ immunized with 300.19 cells expressing L-selectin at their surface. In the ELISA assay used, these antibodies are detectable only if they carry human μ constant region heavy chains.

Figure 6 shows the serum titers of a Xenomouse™ immunized with 300.19 cells expressing L-selectin at their surface. In the ELISA assay used, these antibodies are detectable only if they carry human κ light chains.

Figure 7 shows the serum titers of a Xenomouse™ immunized with 300.19 cells expressing L-selectin. In this ELISA, these antibodies are detectable if they carry human κ light chain and/or murine γ constant regions.

Figure 8 shows a FACS analysis of human neutrophils coupled to sera from a Xenomouse™ (A195-2) immunized with human L-selectin and labeled with an antibody immunoreactive with murine heavy chain γ constant region.

Figure 9 shows a FACS analysis of human neutrophils incubated with serum from a Xenomouse™ (A195-2) immunized with human L-selectin and labeled with an antibody immunoreactive with human light chain κ region.

Figure 10 is a diagram of a plasmid used to transfect mammalian cells to effect the production of the human protein gp39.

Figure 11 represents the serum titration curve of mice immunized with CHO cells expressing human gp39. The antibodies detected in this ELISA must be immunoreactive with gp39 and

contain human heavy chain μ constant regions or human κ light chains.

Figure 12 shows the results of a FACS analysis of antibodies from a Xenomouse™ (labeled A247-4) immunized with human gp39 reacted with activated human T cells. Figure 12A shows the separation of human activated T cells into $CD4^+$ and $CD4^-$ populations. Panel B shows the results of a FACS analysis of the activated $CD4^+$ T cells with antibodies from the Xenomouse™ immunized with gp39 which contain murine heavy chain γ constant regions; panel C shows the corresponding results with respect to $CD4^-$ populations.

Figure 13 is a titration curve with respect to monoclonal antibodies secreted by the hybridoma clone D5.1. This clone is obtained from a Xenomouse™ immunized with tetanus toxin C (TTC) and contains human κ light chain and human μ constant region in the heavy chain.

Figure 14 is a titration curve with respect to the hybridoma supernatant from clone K4.1. This hybridoma clone is obtained from a Xenomouse™ immunized with TTC and contains human κ light chain and heavy chain having the murine γ constant region.

Figure 15 shows binding curves for various concentrations of the K4.1 monoclonal antibody in a determination of the affinity of the monoclonal with its antigen in a BIAcore instrument.

Figure 16 shows the complete nucleotide sequence of the heavy chain from the antibody secreted by K4.1.

Figure 17 shows the complete nucleotide sequence of the light chain from the antibody secreted by K4.1.

Figure 18 shows the complete nucleotide sequence of the heavy chain from the antibody secreted by D5.1.

Figure 19 shows the complete nucleotide sequence of the light chain from the antibody secreted by D5.1.

Modes of Carrying Out the Invention

In general, the methods of the invention include administering an antigen for which human forms of immunospecific reagents are desired to a transgenic nonhuman animal which has

been modified genetically so as to be capable of producing human, but not endogenous, antibodies. Typically, the animal has been modified to disable the endogenous heavy and/or light chain loci in its genome, so that these endogenous loci are incapable of the rearrangement required to generate genes encoding immunoglobulins in response to an antigen. In addition, the animal will have been provided, stably, in its genome, at least one human heavy chain locus and at least one human light chain locus so that in response to an administered antigen, the human loci can rearrange to provide genes encoding human variable regions immunospecific for the antigen.

The details for constructing such an animal useful in the method of the invention are provided in the PCT application WO 94/02602 referenced above.

For production of the desired antibodies, the first step is administration of the antigen. Techniques for such administration are conventional and involve suitable immunization protocols and formulations which will depend on the nature of the antigen per se. It may be necessary to provide the antigen with a carrier to enhance its immunogenicity and/or to include formulations which contain adjuvants and/or to administer multiple injections, and the like. Such techniques are standard and optimization of them will depend on the characteristics of the particular antigen for which immunospecific reagents are desired.

As used herein, the term "immunospecific reagents" includes immunoglobulins and their analogs. The term "analogs" has a specific meaning in this context. It refers to moieties that contain the fully human portions of the immunoglobulin which account for its immunospecificity. In particular, variable regions including the complementarity determining regions (CDRs) are required, along with sufficient portions of the framework regions (FRs) to result in the appropriate three dimensional conformation. Typical immunospecific analogs of antibodies include $F_{(ab')_2}$, $F_{ab'}$, and F_{ab} regions. Modified forms of the variable regions to obtain, for example, single chain F_V analogs with the appropriate immunospecificity are known. A review of such F_V construction is found, for example, in Tibtech

(1991) 2:____. The construction of antibody analogs with multiple immunospecificities is also possible by coupling the human variable regions derived from antibodies with varying specificities.

The variable regions with fully human characteristics can also be coupled to a variety of additional substances which can provide toxicity, biological functionality, alternative binding specificities and the like. The moieties including the fully human variable regions produced by the methods of the invention include single-chain fusion proteins, molecules coupled by covalent methods other than those involving peptide linkages, and aggregated molecules. Examples of analogs which include variable regions coupled to additional molecules covalently or noncovalently include those in the following nonlimiting illustrative list. Traunecker, A. *et al.* Int J Cancer Supp (1992) Supp 7:51-52 describe the bispecific reagent janusin in which the F_V region directed to CD3 is coupled to soluble CD4 or to other ligands such as OVCA and IL-7. Similarly, the fully human variable regions produced by the method of the invention can be constructed into F_V molecules and coupled to alternative ligands such as those illustrated in the cited article. Higgins, P.J. *et al.* J Infect Disease (1992) 166:198-202 describe a heteroconjugate antibody composed of OKT3 cross-linked to an antibody directed to a specific sequence in the V3 region or GP120. Such heteroconjugate antibodies can also be constructed using at least the human variable regions contained in the immunoglobulins produced by the invention methods. Additional examples of bispecific antibodies include those described by Fanger, M.W. *et al.* Cancer Treat Res (1993) 68:181-194 and by Fanger, M.W. *et al.* Crit Rev Immunol (1992) 12:101-124. Conjugates that are immunotoxins including conventional antibodies have been widely described in the art. The toxins may be coupled to the antibodies by conventional coupling techniques or immunotoxins containing protein toxin portions can be produced as fusion proteins. The analogs of the present invention can be used in a corresponding way to obtain such immunotoxins. Illustrative of such immunotoxins are those

described by Byrs, B.S. *et al.* Seminars Cell Biol (1991) 2:59-70 and by Fanger, M.W. *et al.* Immunol Today (1991) 12:51-54.

It will also be noted that some of the immunoglobulins and analogs of the invention will have agonist activity with respect to antigens for which they are immunospecific in the cases wherein the antigens perform signal transducing functions. Thus, a subset of antibodies or analogs prepared according to the methods of the invention which are immunospecific for, for example, a cell surface receptor, will be capable of eliciting a response from cells bearing this receptor corresponding to that elicited by the native ligand. Furthermore, antibodies or analogs which are immunospecific for substances mimicking transition states of chemical reactions will have catalytic activity. Hence, a subset of the antibodies and analogs of the invention will function as catalytic antibodies.

In short, the genes encoding the immunoglobulins produced by the transgenic animals of the invention can be retrieved and the nucleotide sequences encoding the fully human variable region can be manipulated according to known techniques to provide a variety of analogs such as those described above. In addition, the immunoglobulins themselves containing the human variable regions can be modified using standard coupling techniques to provide conjugates retaining immunospecificity and fully human characteristics in the immunospecific region.

Thus, immunoglobulin "analogs" refers to moieties which contain those portions of the antibodies of the invention which retain their human characteristics and their immunospecificity. These will retain sufficient human variable region to provide the desired specificity.

As stated above, all of the methods of the invention include administering the appropriate antigen to the transgenic animal. The recovery or production of the antibodies themselves can be achieved in various ways.

First, and most straightforward, the polyclonal antibodies produced by the animal and secreted into the bloodstream can be recovered using known techniques. Purified forms of these antibodies can, of course, be readily prepared by standard purification techniques, preferably including affinity

chromatography with respect to the particular antigen, or even with respect to the particular epitope of the antigen for which specificity is desired. In any case, in order to monitor the success of immunization, the antibody levels with respect to the antigen in serum will be monitored using standard techniques such as ELISA, RIA and the like.

It will be noted, from the examples below, that a portion of the polyclonal antiserum obtained may include an endogenous heavy chain constant region derived from the host, even though the variable regions are fully human. Under these circumstances, to the extent that an application requires fully human antibodies, use of the polyclonal antiserum directly would be inappropriate. However, the presence of these chimeras, which is believed to result from *in vivo* isotype switching as described by Gerstein *et al.* Cell (1990) 63:537, is not problematic, in view of conventional purification and modification methods and in view of the availability of alternative methods to recover fully human antibodies, if desired, described in the following paragraphs.

First, and most simply, the polyclonal antiserum could be subjected to suitable separation techniques to provide compositions containing only fully human immunoglobulins. Portions of the serum which display characteristics of the host species can be removed, for example, using affinity reagents with the appropriate anti species immunoglobulins or immunospecific portions thereof. Furthermore, for applications where only the variable regions of the antibodies are required, treating the polyclonal antiserum with suitable reagents so as to generate F_{ab}, $F_{ab'}$, or $F_{(ab')_2}$ portions results in compositions containing fully human characteristics. Such fragments are sufficient for use, for example, in immunodiagnostic procedures involving coupling the immunospecific portions of immunoglobulins to detecting reagents such as radioisotopes. Thus, for some applications, the polyclonal antiserum can be treated to provide compositions with the desired characteristics including compositions consisting essentially of fully human antibodies and compositions including immunoglobulin analogs wherein the immunospecific portion is fully human.

Alternatively, immunoglobulins and analogs with desired characteristics can be generated from immortalized B cells derived from the transgenic animals used in the method of the invention or from the rearranged genes provided by these animals in response to immunization. It will be apparent that hybridomas derived from the B cells of the immunized animal can be screened so as to choose only those secreting fully human antibodies and that the genetic material can be recovered from the hybridomas or from lymphocytes in spleen, blood, or lymph nodes of the immunized animal and manipulated using conventional techniques to replace any endogenous constant region with a human one or to produce a desired analog.

Thus, as an alternative to harvesting the antibodies directly from the animal, the B cells can be obtained, typically from the spleen, but also, if desired, from the peripheral blood lymphocytes or lymph nodes and immortalized using any of a variety of techniques, most commonly using the fusion methods described by Kohler and Milstein. The resulting hybridomas (or otherwise immortalized B cells) can then be cultured as single colonies and screened for secretion of antibodies of the desired specificity. As described above, the screen can also include a determination of the fully human character of the antibody. For example, as described in the examples below, a sandwich ELISA wherein the monoclonal in the hybridoma supernatant is bound both to antigen and to an antihuman constant region can be employed. Conversely, hybridomas that secrete antibodies which are immunoreactive with antispecies antibodies directed to the species of the immunized animal can be discarded. After the appropriate hybridomas are selected, the desired antibodies can be recovered, again using conventional techniques. They can be prepared in quantity by culturing the immortalized B cells using conventional methods, either *in vitro*, or *in vivo* to produce ascites fluid. Purification of the resulting monoclonal antibody preparations is less burdensome than in the case of serum since each immortalized colony will secrete only a single type of antibody. In any event, standard purification techniques to isolate the antibody from other proteins in the culture medium can be employed.

As an alternative to obtaining human immunoglobulins directly from the culture of immortalized B cells derived from the animal, the immortalized cells can be used as a source of rearranged heavy chain and light chain loci for subsequent expression and/or genetic manipulation. Isolation of genes from such antibody-producing cells is straightforward since high levels of the appropriate mRNAs are available for production of a cDNA library. The recovered rearranged loci can be manipulated as desired. For example, the constant region can be exchanged for that of a different isotype or that of a human antibody, as described above, or eliminated altogether. The variable regions can be linked to encode single chain F_V regions. Multiple F_V regions can be linked to confer binding ability to more than one target or chimeric heavy and light chain combinations can be employed. Once the genetic material is available, design of analogs as described above which retain their ability to bind the desired target, as well as their human characteristics, is straightforward.

Once the appropriate genetic material is obtained and, if desired, modified to encode an analog, the coding sequences including those that encode, at a minimum, the variable regions of the human heavy and light chain can be inserted into expression systems contained on vectors which can be transfected into standard recombinant host cells. As described below, a variety of such host cells may be used; for efficient processing, however, mammalian cells are preferred. Typical mammalian cell lines useful for this purpose include CHO cells, 293 cells, or NSO-GS cells.

The production of the antibody or analog is then undertaken by culturing the modified recombinant host under culture conditions appropriate for the growth of the host cells and the expression of the coding sequences. The antibodies are then recovered from the culture. The expression systems are preferably designed to include signal peptides so that the resulting antibodies are secreted into the medium; however, intracellular production is also possible.

In addition to deliberate design of modified forms of the immunoglobulin genes to produce analogs, advantage can be

taken of phage display techniques to provide libraries containing a repertoire of antibodies with varying affinities for the desired antigen. For production of such repertoires, it is unnecessary to immortalize the B cells from the immunized animal; rather the primary B cells can be used directly as a source of DNA. The mixture of cDNAs obtained from B cells, e.g., derived from spleens, is used to prepare an expression library, for example, a phage display library transfected into *E. coli*. The resulting cells are tested for immunoreactivity to the desired antigen. Techniques for the identification of high affinity human antibodies from such libraries are described by Griffiths, A.D., *et al.*, EMBO J (1994) 13:3245-3260; by Nissim, A., *et al. ibid*, 692-698, and by Griffiths, A.D., *et al.*, *ibid*, 725-734. Ultimately, clones from the library are identified which produce binding affinities of a desired magnitude for the antigen, and the DNA encoding the product responsible for such binding is recovered and manipulated for standard recombinant expression. Phage display libraries may also be constructed using previously manipulated nucleotide sequences and screened in similar fashion. In general, the cDNAs encoding heavy and light chain are independently supplied or are linked to form F_V analogs for production in the phage library.

The phage library is thus screened for the antibodies with highest affinity for the antigen and the genetic material recovered from the appropriate clone. Further rounds of screening can increase the affinity of the original antibody isolated. The manipulations described above for recombinant production of the antibody or modification to form a desired analog can then be employed.

As above, the modified or unmodified rearranged loci are manipulated using standard recombinant techniques by constructing expression systems operable in a desired host cell, such as, typically, a Chinese hamster ovary cell, and the desired immunoglobulin or analog is produced using standard recombinant expression techniques, and recovered and purified using conventional methods.

The application of the foregoing processes to antibody production has enabled the preparation of human immunospecific

reagents with respect to antigens for which human antibodies have not heretofore been available. The immunoglobulins that result from the above-described methods and the analogs made possible thereby, provide novel compositions for use in analysis, diagnosis, research, and therapy. The particular use will, of course, depend on the immunoglobulin or analog prepared. In general, the compositions of the invention will have utilities similar to those ascribable to nonhuman antibodies directed against the same antigen. Such utilities include, for example, use as a affinity ligands for purification, as reagents in immunoassays, as components of immunoconjugates, and as therapeutic agents for appropriate indications.

Particularly in the case of therapeutic agents or diagnostic agents for use *in vivo*, it is highly advantageous to employ antibodies or their analogs with fully human characteristics. These reagents avoid the undesired immune responses engendered by antibodies or analogs which have characteristics marking them as originating from non-human species. Other attempts to "humanize" antibodies do not result in reagents with fully human characteristics. For example, chimeric antibodies with murine variable regions and human constant regions are easily prepared, but, of course, retain murine characteristics in the variable regions. Even the much more difficult procedure of "humanizing" the variable regions by manipulating the genes encoding the amino acid sequences that form the framework regions does not provide the desired result since the CDRs, typically of nonhuman origin, cannot be manipulated without destroying immunospecificity. Thus, the methods of the present invention provide, for the first time, immunoglobulins that are fully human or analogs which contain immunospecific regions with fully human characteristics.

There are large numbers of antigens for which human antibodies and their human analogs would be made available by the methods of the invention. These include the following as a nonlimiting set:

leukocyte markers, such as CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD11a,b,c, CD13, CD14, CD18, CD19, CD20, CD22, CD23,

CD27 and its ligand, CD28 and its ligands B7.1, B7.2, B7.3, CD29 and its ligand, CD30 and its ligand, CD40 and its ligand gp39, CD44, CD45 and isoforms, CDw52 (Campath antigen), CD56, CD58, CD69, CD72, CTLA-4, LFA-1 and TCR

histocompatibility antigens, such as MHC class I or II, the Lewis Y antigens, SLex, SLey, SLea, and SLeb;

adhesion molecules, including the integrins, such as VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, LFA-1, Mac-1 and p150,95; and

the selectins, such as L-selectin, E-selectin, and P-selectin and their counterreceptors VCAM-1, ICAM-1, ICAM-2, and LFA-3;

interleukins, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15;

interleukin receptors, such as IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-8R, IL-9R, IL-10R, IL-11R, IL-12R, IL-13R, IL-14R, and IL-15R;

chemokines, such as PF4, RANTES, MIP1α, MCP1, NAP-2, Groα, Groβ, and IL-8;

growth factors, such as TNFalpha, TGFbeta, TSH, VEGF/VPF, PTHrP, EGF family, FGF, PDGF family, endothelin, and gastrin releasing peptide (GRP);

growth factor receptors, such as TNFalphaR, RGFbetaR, TSHR, VEGFR/VPFR, FGFR, EGFR, PTHrPR, PDGFR family, EPO-R, GCSF-R and other hematopoietic receptors;

interferon receptors, such as IFNαR, IFNβR, and IFNγR;

Igs and their receptors, such as IgE, FceRI, and FCeRII;

tumor antigens, such as her2-neu, mucin, CEA and endosialin;

allergens, such as house dust mite antigen, lol p1 (grass) antigens, and urushiol;

viral proteins, such as CMV glycoproteins B, H, and gCIII, HIV-1 envelope glycoproteins, RSV envelope glycoproteins, HSV envelope glycoproteins, EBV envelope glycoproteins, VZV envelope glycoproteins, HPV envelope glycoproteins, Hepatitis family surface antigens;

toxins, such as pseudomonas endotoxin and osteopontin/uropontin, snake venom, and bee venom;

blood factors, such as complement C3b, complement C5a, complement C5b-9, Rh factor, fibrinogen, fibrin, and myelin associated growth inhibitor;

enzymes, such as cholesterol ester transfer protein, membrane bound matrix metalloproteases, and glutamic acid decarboxylase (GAD); and

miscellaneous antigens including ganglioside GD3, ganglioside GM2, LMP1, LMP2, eosinophil major basic protein, eosinophil cationic protein, pANCA, Amadori protein, Type IV collagen, glycated lipids, γ-interferon, A7, P-glycoprotein and Fas (AFO-1) and oxidized-LDL.

Particularly preferred immunoglobulins and analogs are those immunospecific with respect to human IL-6, human IL-8, human TNFα, human CD4, human L-selectin, and human gp39. Human antibodies against IL-8 are particularly useful in preventing tumor metastasis and inflammatory states such as asthma and reperfusion injury. Antibodies and analogs immunoreactive with human TNFα and human IL-6 are useful in treating cachexia and septic shock as well as autoimmune disease. Antibodies and analogs immunoreactive with gp39 or with L-selectin are also effective in treating or preventing autoimmune disease. In addition, anti-gp39 is helpful in treating graft versus host disease, in preventing organ transplant rejection, and in treating glomerulonephritis. Antibodies and analogs against L-selectin are useful in treating ischemia associated with reperfusion injury.

Typical autoimmune diseases which can be treated using the above-mentioned antibodies and analogs include systemic lupus erythematosus, rheumatoid arthritis, psoriasis, Sjogren's, scleroderma, mixed connective tissue disease, dermatomyositis, polymyositis, Reiter's syndrome, Behcet's disease, Type 1 diabetes, Hashimoto's thyroiditis, Grave's disease, multiple sclerosis, myasthenia gravis and pemphigus.

The examples below are intended to illustrate but not to limit the invention.

In these examples, mice, designated "xenomice", are used for initial immunizations. A detailed description of such xenomice is found in the above referenced PCT application WO 94/02602. Immunization protocols appropriate to each antigen are described in the specific examples below. The sera of the immunized xenomice (or the supernatants from immortalized B cells) were titrated for antigen specific human antibodies in each case using a standard ELISA format. In this format, the antigen used for immunization was immobilized onto wells of microtiter plates. The plates were washed and blocked and the sera (or supernatants) were added as serial dilutions for 1-2 hours of incubation. After washing, bound antibody having human characteristics was detected by adding the appropriate antispecies Ig (typically antihuman κ chain antibody or antihuman μ chain antibody) conjugated to horseradish peroxidase (HRP) for one hour. In some cases, the bound antibodies were tested for murine characteristics using antimurine antibodies, typically antimurine γ chain antibody. After again washing, the chromogenic reagent o-phenylene diamine (OPD) substrate and hydrogen peroxide were added and the plates were read 30 minutes later at 492 nm using a microplate reader.

Unless otherwise noted, the antigen was coated using plate coating buffer (0.1 M carbonate buffer, pH 9.6); the assay blocking buffer used was 0.5% BSA, 0.1% Tween 20 and 0.01% Thimerosal in PBS; the substrate buffer used in color development was citric acid 7.14 g/l: dibasic sodium phosphate 17.96 g/l; the developing solution (made immediately before use) was 10 ml substrate buffer, 10 mg OPD, plus 5 ml hydrogen peroxide; the stop solution (used to stop color development) was 2 M sulfuric acid. The wash solution was 0.05% Tween 20 in PBS.

Example 1

Human Antibodies Against Human IL-6

Three to 5 xenomice aged 8-20 weeks were age-matched and immunized intraperitoneally with 50 μg human IL-6 emulsified in complete Freund's adjuvant for primary immunization and in incomplete Freund's adjuvant for subsequent injections. The mice received 6 injections 2-3 weeks apart. Serum titers were

determined after the second dose and following each dose thereafter. Bleeds were performed 6-7 days after injections from the retrobulbar plexus. The blood was allowed to clot at room temperature for about 2 hours and then incubated at 4°C for at least 2 hours before separating and collecting the sera.

ELISAs were conducted as described above by applying 100 μl/well of recombinant human IL-6 at 2 mg/ml in coating buffer. Plates were then incubated at 4°C overnight or at 37°C for 2 hours and then washed three times in washing buffer. Addition of 100 μl/well blocking buffer was followed by incubation at room temperature for 2 hours, and an additional 3 washes.

Then, 50 μl/well of diluted serum samples (and positive and negative controls) were added to the plates. Plates were then incubated at room temperature for 2 hours and again washed 3 times.

After washing, 100 μl/well of either mouse antihuman μ chain antibody conjugated to HRP at 1/2,000 or mouse antihuman κ chain antibody conjugated to HRP at 1/2,000, diluted in blocking buffer were added. After a 1 hour incubation at room temperature, the plates were washed 3 times and developed with OPD substrate for 10-25 minutes. 50 μl/well of stop solution were then added and the results read on an ELISA plate reader at 492 nm. The dilution curves resulting from the titration of serum from Xenomouse™ A40-7 after 6 injections are shown in Figure 1. The data in Figure 1 show production of anti-IL-6 immunoreactive with antihuman κ and antihuman μ detectable at serum dilutions above 1:1,000.

Example 2

Human Antibodies Against Human IL-8

Immunization and serum preparation were as described in Example 1 as except that human recombinant IL-8 was used as an immunogen.

ELISA assays were performed with respect to the recovered serum, also exactly as described in Example 1, except that the ELISA plates were initially coated using 100 μl/well of recombinant human IL-8 at 0.5 mg/ml in the coating buffer. The

results obtained for various serum dilutions from Xenomouse™ A260-5 after 6 injections are shown in Figure 2. Human anti-IL-8 binding was again shown at serum dilutions having concentrations higher than that represented by a 1:1,000 dilution.

Example 3

Human Antibodies Against Human TNFα

Immunization and serum preparation were conducted as described in Example 1 except that human recombinant TNFα was substituted for human IL-6. ELISAs were conducted as described in Example 1 except that the initial coating of the ELISA plate employed 100 µl/well recombinant human TNFα at 1 mg/ml in coating buffer.

The dilution curves for serum from Xenomouse™ A210-8 after 6 injections obtained are shown in Figure 3. Again significant titers of human anti-TNFα binding were shown.

Example 4

Human Antibodies Against Human CD4

The human CD4 antigen was prepared as a surface protein using human CD4 ζ on transfected recombinant cells as follows. Human CD4 ζ consists of the extracellular domain of CD4, the transmembrane domain of CD4, and the cytoplasmic domain of residues 31-142 of the mature ζ chain. Human CD4 zeta (F15 LTR) as described in Roberts, *et al.*, Blood (1994) 84:2878 was introduced into the rat basophil leukemic cell line RBL-2H3, described by Callan, M., *et al.*, Proc Natl Acad Sci USA (1993) 90:10454 using the *kat* high efficiency transduction system described by Finer, *et al.*, Blood (1994) 83:43. Briefly, RBL-2H3 cells at 10^6 cells per well were cultured in 750 ml $DMEM^{low}$ + 20% FBS (Gibco) and 16 µg/ml polybrene with an equal volume of proviral supernatant for 2 hours at 37°C, 5% CO_2. One ml of medium was removed and 750 µl of infection medium and retroviral supernatant were added to each well and the cultures incubated overnight. The cells were washed and expanded in $DMEM^{low}$ + 10% FBS until sufficient cells were available for sorting. The $CD4^-$ zeta transduced RBL-2H3 cells were sorted using the FACSTAR plus

(Becton Dickinson). The cells were stained for human CD4 with a mouse antihuman CD4⁻ PE antibody and the top 2-3% expressing cells were selected.

Immunizations were conducted as described in Example 1 using 10 x 10^6 cells per mouse except that the primary injection was subcutaneous at the base of the neck. The mice received 6 injections 2-3 weeks apart. Serum was prepared and analyzed by ELISA as described in Example 1 except that the initial coating of the ELISA plate utilized 100 µl per well of recombinant soluble CD4 at 2 mg/ml of coating buffer. The titration curve for serum from Xenomouse™ A207-1 after 6 injections is shown in Figure 4. Titers of human anti-CD4 reactivity were shown at concentrations representing greater than those at 1:1,000 dilution.

Example 5

Human Antibodies Against Human L-selectin

The antigen was prepared as a surface displayed protein in C51 cells, a high expressing clone derived by transfecting the mouse pre-B cell 300.19 with LAM-1 cDNA (LAM-1 is the gene encoding L-selectin) (Tedder, *et al.*, J Immunol (1990) 144:532) or with similarly transfected CHO cells. The transfected cells were sorted using fluorescent activated cell sorting using anti-Leu-8 antibody as label.

The C51 and the transfected CHO cells were grown in DME 4.5 g/l glucose with 10% FCS and 1 mg/ml G418 in 100 mm dishes. Negative control cells, 3T3-P317 (transfected with gag/pol/env genes of Moloney virus) were grown in the same medium without G418.

Primary immunization was done by injection subcutaneously at the base of the neck; subsequent injections were intraperitoneal. 70-100 million C51 or transfected CHO cells were used per injection for a total of five injections 2-3 weeks apart.

Sera were collected as described in Example 1 and analyzed by ELISA in a protocol similar to that set forth in Example 1.

For the ELISA, the transfected cells were plated into 96 well plates and cell monolayers grown for 1-2 days depending on cell number and used for ELISA when confluent. The cells were fixed by first washing with cold 1 x PBS and then fixing solution (5% glacial acetic acid, 95% ethanol) was added. The plates were incubated at -25°C for 5 minutes and can be stored at this temperature if sealed with plate sealers.

The ELISA is begun by bringing the plates to room temperature, flicking to remove fixing solution and washing 5 times with DMEM medium containing 10% FCS at 200 µl per well.

The wells were treated with various serum dilutions or with positive or negative controls. Positive control wells contained murine IgG1 monoclonal antibody to human L-selectin.

The wells were incubated for 45 minutes and monolayer integrity was checked under a microscope. The wells were then incubated with either antimouse IgG (1/1000) or with antihuman κ chain antibody or antihuman µ chain antibody conjugates with HRP described in Example 1. The plates were then washed with 1% BSA/PBS and again with PBS and monolayer integrity was checked. The plates were developed, stopped, and read as described above. The results for serum from Xenomouse™ A303-3 are shown in Figs. 5 and 6; human antibodies both to L-selectin and control 3T3 cells were obtained. However, the serum titers are higher for the L-selectin-expressing cells as compared to parental 3T3 cells. These results show that Xenomouse™ A303-3 produces antibodies specific for L-selectin with human µ heavy chain regions and/or human κ light chains.

ELISAs were also performed using as the immobilized antigen a fusion protein consisting of the extracellular domain of human L-selectin fused to the constant domain of human IgG_1 (Guo, *et al.*, Cell Immunol (1994) 154:202). The L-selectin fusion protein was made by transient transfection of human 293 cells using calcium phosphate transfection (Wigler, M., Cell (1979) 16:777). Serum preparation was performed as described in Example 1. ELISAs were conducted essentially as in Example 1, except that the initial coating of the ELISA plate employed 100 µl transfected 293 cell culture supernatant containing the

L-selectin-Ig fusion protein. Detection employed HRP-mouse antihuman κ and HRP-goat antimouse IgG.

Figure 7 shows the results from Xenomouse™ A195-2; antibodies specific for L-selectin having human κ light chains and/or human variable regions with murine heavy chain γ regions are present in the serum.

The antisera obtained from the immunized xenomice were also tested for staining of human neutrophils which express L-selectin. Human neutrophils were prepared as follows: peripheral blood was collected from normal volunteers with 100 units/ml heparin. About 3.5 ml blood was layered over an equal volume of One-step Polymorph Gradient (Accurate Chemical, Westbury, NY) and spun for 30 minutes at 450 x g at 20°C. The neutrophil fraction was removed and washed twice in DPBS/2% FBS.

The neutrophils were then stained with either:

(1) antiserum from Xenomouse™ A195-2 immunized with C51 cells (expressing L-selectin);

(2) as a positive control, mouse monoclonal antibody LAM1-3 (against L-selectin); and

(3) as negative control, antiserum from a Xenomouse™ immunized with cells expressing human gp39.

The stained, washed neutrophils were analyzed by FACS. The results for antiserum from Xenomouse™ A195-2 are shown in Figures 8 and 9.

These results show the presence of antibodies in immunized Xenomouse™ serum which contain fully human variable regions immunoreactive with L-selectin. The negative control antiserum from mice immunized with gp39 does not contain antibodies reactive against human neutrophils. Serum from A195-2 (immunized with L-selectin-expressing cells) contains antibodies binding to human neutrophils detectable with a goat antimouse IgG antibody (Figure 8), which binds with heavy chain protein composed of fully human variable regions and mouse γ constant regions. Staining with anti L-selectin Xenomouse™ antisera detected with a mouse monoclonal antibody against human κ chain antibody is shown in Figure 9, showing the presence of fully human κ light chain.

As explained above, these antibodies containing human variable regions are readily convertible to fully human antibodies. For example, using hybridomas secreting these antibodies, the cDNAs encoding them can be obtained. By amplifying the genes encoding human V regions using primers containing restriction enzyme recognition sites and cloning them into plasmids containing the coding sequences for human constant regions as described by Queen, *et al.*, Proc Natl Acad Sci (1989) 86:10029, genes encoding the fully human antibodies can be obtained for recombinant production.

Example 6

Human Antibodies Against Human gp39

gp39 (the ligand for CD40) is expressed on activated human $CD4^+$ T cells. The sera of xenomice immunized with recombinant gp39 according to this example contained antibodies immunospecific for gp39 with fully human variable regions; the sera contained fully human IgM antibodies and chimeric IgG antibodies containing human variable regions and murine constant heavy chain γ region.

The antigen consisted of stable transfectants of 300.19 cells or of CHO cells expressing gp39 cDNA cloned into the mammalian expression vector P1K1.HUgp39/IRES NEO as shown in Figure 10. CHO cells were split 1:10 prior to transfection in DMEM 4.5 g/l glucose, 10% FBS, 2 mM glutamine, MEM, NEAA supplemented with additional glycine, hypoxanthine and thymidine. The cells were cotransfected with the gp39 vector at 9 μg/10 cm plate (6 X 10^5 cells) and the DHFR expressing vector pSV2DHFRs (Subranani *et al.* Mol Cell Biol (1981) 9:854) at 1 μg/10 cm plate using calcium phosphate transfection. 24 hours later the cells were split 1:10 into the original medium containing G418 at 0.6 mg/ml. Cells producing gp39 were sorted by FACS using an anti-gp39 antibody.

Mice grouped as described in Example 1 were immunized with 300.19 cells expressing gp39 using a primary immunization subcutaneously at the base of the neck and with secondary intraperitoneal injections every 2-3 weeks. Sera were harvested as described in Example 1 for the ELISA assay. The ELISA

procedure was conducted substantially as set forth in Example 1; the microtiter plates were coated with CHO cells expressing gp39 grown in a 100 mm dish in DMEM, 4.5 g/l glucose, 10% FCS, 4 mM glutamine, and nonessential amino acid (NEAA) solution for MEM (100X). On the day preceding the ELISA assay, the cells were trypsinized and plated into 96-well filtration plates at 10^5 cells/200 μl well and incubated at 37°C overnight. The positive controls were mouse antihuman gp39; negative controls were antisera from mice immunized with an antigen other than gp39. 50 μl of sample were used for each assay. The remainder of the assay is as described in Example 1.

The dilution curves for the sera obtained after 4 injections from mice immunized with gp39 expressed on CHO cells are shown in Figure 11. As shown, the sera contained antihuman gp39 immunospecificity which is detectable with human κ and human μ chain antibodies coupled to HRP.

In addition, the sera were tested for their ability to react with activated human T cells included in PBMC using FACS analysis. To prepare the PBMC, human peripheral blood was collected from normal volunteers with the addition of 100 unit/ml heparin. PBMC were isolated over Ficoll gradient and activated with 3 μg/ml PHA, 1 μg/ml PMA in IMDM plus 10% FBS plus 25 μM 2-mercaptoethanol for 4 hours. After washing, the PBMC were stained with mouse Mab against human CD4 labeled with FITC to permit separation of $CD4^+$ and $CD4^-$ human T cells.

The activated $CD4^+$ and $CD4^-$ T cells were then analyzed by FACS using staining with either:

1) antiserum from a Xenomouse™ immunized with 300.19 cells producing gp39;

2) a positive control mouse Mab directed against α-CD40L (human gp39); and

3) a negative control antiserum from a Xenomouse™ immunized with TNF.

The detecting antibody in the FACS analysis was goat antimouse IgG (PE). The results are shown in Figure 12.

As shown in Figure 12A, $CD4^+$ (R2) and $CD4^-$ (R3) cells were separated prior to FACS analysis. Panel B shows the results for $CD4^+$ cells and shows that sera from mice immunized

with gp39 (labeled A247-4 in the figure) reacted with these activated $CD4^+$ T cells; panel C shows that these sera did not react with $CD4^-$ cells. These antibodies carried murine heavy chain γ constant regions. The results of panels B and C also confirm that the TNF-injected Xenomouse™ did not make antibodies against gp39.

Example 7

Preparation of High-Affinity Human Mabs Against Tetanus Toxin

The antibodies prepared in this example were secreted by hybridomas obtained by immortalizing B cells from xenomice immunized with tetanus toxin. The immunization protocol was similar to that set forth in Example 1 using 50 μg tetanus toxin emulsified in complete Freund's adjuvant for intraperitoneal primary immunization followed by subsequent intraperitoneal injections with antigen incorporated into incomplete Freund's adjuvant. The mice received a total of 4 injections 2-3 weeks apart.

After acceptable serum titers of antitetanus toxinC (anti-TTC) were obtained, a final immunization dose of antigen in PBS was given 4 days before the animals were sacrificed and the spleens were harvested for fusion.

The spleen cells were fused with myeloma cells P3X63-Ag8.653 as described by Galfre, G. and Milstein, C. Methods in Enzymology (1981) 73:3-46.

After fusion the cells were resuspended in DMEM, 15% FCS, containing HAT supplemented with glutamine, pen/strep for culture at 37°C and 10% CO_2. The cells were plated in microtiter trays and maintained in HAT-supplemented medium for two weeks before transfer to HAT-supplemented medium. Supernatants from wells containing hybridomas were collected for a primary screen using an ELISA.

The ELISA was conducted as described in Example 1 wherein the antigen coating consisted of 100 μl/well of tetanus toxin C (TTC) protein at 2 mg/ml in coating buffer, followed by incubation at 4°C overnight or at 37°C for two hours. In the primary ELISA, HRP-conjugated goat antimouse IgG at 1/2000 was

used in addition to HRP mouse antihuman IgM as described in Example 1. Two hybridomas that secreted anti-TTC according to the ELISA assay, clone D5.1 and clone K4.1 were used for further analysis.

As shown in Figure 13, clone D5.1 secretes fully human anti-TTC which is detectable using HRP-conjugated antihuman μ chain antibody and HRP-conjugated antihuman κ chain antibody. This is confirmed in Figures 18 and 19. Figure 14 shows that clone K4.1 secretes anti-TTC which is immunoreactive with antimurine γ and antihuman κ HRP-conjugated antibodies. Thus, clone K4.1 provides anti-TTC fully with human variable region as confirmed in Figures 16 and 17 and a murine constant heavy chain γ region.

The antibodies secreted by D5.1 and K4.1 did not immunoreact in ELISAs using TNFα, IL-6, or IL-8 as immobilized antigen under conditions where positive controls (sera from xenomice immunized with TNFα, IL-6 and IL-8 respectively) showed positive ELISA results.

The affinity of the monoclonal antibodies secreted by K4.1 for TTC antigen was determined using commercially available reagents and instrumentation. BIAcore Instrument, CM5 sensor chips, surfactant P20 and the amine coupling kit were purchased from Pharmacia Biosensor (Piscataway, NJ). TTC was immobilized at two levels of antigen density on the surface of the sensor chips according to the manufacturer's instructions. Briefly, after washing and equilibrating the instrument with buffer containing surfactant, the surfaces were activated and the TCC was immobilized.

For high antigen density, the surface was activated with 35 μl of equal volumes 0.1 M NHS and 0.1 M EDC injected across the surface followed by 30 μl of TTC fragment at 100 μg/ml in 10 mM sodium acetate buffer pH 5.0. The surface was blocked by injecting 35 μl 1 M ethanolamine and washed to remove noncovalently bound TCC using 5 μl 0.1 M HCl. The entire immobilization procedure was conducted with a continuous flow of buffer at 5 μl/min. This results in about 7500-8500 response units (RU) of TTC per chip. (1000 RU corresponds to about 1 ng of protein per mm^2.)

For chips with low antigen density, the procedure utilizes 15 μl rather than 30 μl of TTC, resulting in chips containing 550-950 RU.

Chips could be regenerated after use in single determinations by injecting 10 μl formal or $MgCl_2$.

The chips are used to determine binding affinities by determining k_a and k_b (the association and dissociation rate constants) for the antibody with respect to the immobilized TTC. The association rate constant is measured over six minutes at a flow rate of 5 μl/min. at different concentrations of K4.1 Mab in the range of 2.16 nm-69.33 nm. The dissociation rate constant is measured at a constant buffer flow rate of 5 μl/min after completion of the antibody injection. The raw data are graphed in Figure 15 and the calculated results are shown in Table 1.

Table 1
Kinetic Constants of K4.1 Measured Using the BIAcore on Two Different Surfaces

Immobilized tetanus toxinC	K4.1 conc. range nM	Association rate ka(10^5 $M^{-1}s^{-1}$)	Dissociation rate kd($10^5 s^{-1}$)	Binding constant KA(M^{-1})=ka/kd	Dissociation constant KD(M)=kd/ka
931 RU	4.3 - 34.7	6.47 ± 1.05	4.02 ± 1.42	1.6 X 10^{10}	0.62 X 10^{-10}
868 RU	4.3 - 34.7	7.19 ± 2.18	2.02 ± 1.01	3.5 x 10^{10}	0.28 x 10^{-10}

As shown, the K4.1 antibody has a binding constant (K_a) for TTC somewhat larger than 10^{10} M^{-1}.

The complete nucleotide sequence of the cDNAs encoding the heavy and light chains of the K4.1 and D5.1 monoclonals were determined as shown in Figures 16-19. PolyA mRNA was isolated from about 10^6 hybridoma cells and used to generate cDNA using random hexamers as primers. Portions of the product were amplified by PCR using the appropriate primers.

Both cell lines were known to provide human κ light chains; for PCR amplification of light chain encoding cDNA, the primers used were HKP1 (5'-CTCTGTGACACTCTCCTGGGAGTT-3') for priming from the constant region terminus and two oligos, used in equal amounts to prime from the variable segments: B3 (5'-CCACCATCAACTGCAAGTCCAGCCA-3') and B2/B1 (5'-GAAACGACACTCACGCAGTCTCCAGC-3').

For amplification of the heavy chain from K4.1 (which contains the murine γ1 constant region), the primers were MG-24Vi for the human variable regions: 5'-CAGGTGCAGCTGGAGCAGTCiGG-3' which, with inosine as shown recognizes the human variable regions V_{H1-2}, V_{H1-3}, V_{H4} and V_{H6}, and from the constant region MG-25 i.e., 5'-GCACACCGCTGGACAGGGATCCAiAGTTTC-3', which, containing inosine as shown recognizes murine γ1, γ2A, γ2B, and γ3.

For amplification of the heavy chain of the antibody derived from D5.1 (which contains the human μ constant region), MG-24VI was used to prime from the variable and μP1 (5'-TTTTCTTTGTTGCCGTTGGGGTGC-3') was used to prime from the constant region terminus.

Turning first to the results shown in Figure 16 representing the heavy chain of the Mab secreted by K4.1, the sequence shows the presence of the human variable segment VH6, the human diversity region DN1, and the human joining segment JH4 linked to the murine γ1 constant region. Nine base-pair mutations from the published germline sequence were present in the variable region, two of them within CDR2. One mutation was observed in the D segment. Three nongermline nucleotide additions were present in the D_H-J_H junction.

Referring to Figure 17 which shows the light chain of the K4.1 antibody, analysis shows the presence of the human κ variable region B3 and joining region JK4. Eight nucleotides are missing from B3 at the V_K-J_K junction and four mutations were found in the variable region. Five nongermline nucleotide additions were present at the V_K-J_K junction.

Referring now to Figure 18 which sets forth the sequence for the heavy chain of the antibody secreted by clone D5.1, this shows the heavy chain is comprised of the human variable fragment VH6, the human diversity region DN1 and the human joining segment JH4 linked to the human μ constant region. There were two base-pair mutations from the germline sequence in the variable region, neither within the CDRs. Two additional mutations were in the D segment and six nongermline nucleotide additions were present at the D_H-J_H junction.

Finally, referring to Figure 19 which presents the light chain of the antibody secreted by D5.1, the human κ variable region B3 and human κ joining region JK3 are shown. There are nine base-pair differences from the germline sequences, three falling within CDR1.

Example 8

Production of Human Antibodies to IgE

A. Immunization of Mice

Germline chimeric mice containing integrated human DNA from the immunoglobulin loci were immunized by injection of 15-20 μg of human IgE/λ in adjuvant. The mice were boosted with 15-20 μg of human IgE/λ every 14 days after the primary immunization. A bleed was done on the immunized animals to test the titer of serum antibodies against human IgE/λ. The mice with the highest titers were sacrificed and the spleen removed.

B. Fusion of Splenocytes

Myeloma cells, line P3X63-Ag8.653, used as the fusion partner for the spleen cells, were thawed 6 days prior to the fusion and grown in tissue culture. One day before the fusion, cells were split into fresh medium containing 10% fetal calf serum (FCS) at a ratio of 1:3.

After sacrificing the mouse, the spleen was aseptically removed and placed in a culture dish with serum-free culture medium. A single cell suspension was created by gently grinding the spleen between two frosted microscope slides. The cells were washed in fresh serum-free medium red blood cells were lysed and debris filtered away.

The splenocytes were further washed twice by centrifugation in serum-free medium. Myeloma cells were also washed in serum-free medium at this time. Each cell type was counted and combined at a ratio of 1:3 (myeloma to splenocyte), mixed gently and centrifuged once together.

A solution of 40% polyethylene glycol (PEG) was slowly added to the cell pellet while the cells were gently resuspended over a period of one minute. Cells were incubated at room temperature for one minute in the PEG solution and then slowly diluted into 5 ml serum-free medium over 5 minutes. Five ml more were added over the next 90 seconds. Cells were incubated at room temperature for 5 minutes. The cells were centrifuged at low speed and the supernatant removed. The cells were resuspended slowly and very gently in 5 ml of hybridoma medium containing 10% FCS, 1X OPI, 1X NE amino acids and 10 mM HEPES. Cells were further diluted to 100 ml final volume in hybridoma medium with 1X HAT solution (hypoxanthine, aminopterin and thymidine). The fused cells were aliquoted 100 µl/well of 96-2311 plates and cultured at 37°C and 10% CO_2. Cells were fed at 10 days post-fusion with 100 µl/well of hybridoma medium with 1X HT (hypoxanthine and thymidine) and allowed to grow close to confluence before screening.

Supernatant was aseptically taken from each growing well and tested for the presence of fully human antibodies. Positive wells were further tested for human IgE/λ specificity. When a positive well was identified, the cells were transferred from the 96-well plate to 0.5 ml of hybridoma medium with 1X HT in a 48-well plate. At this stage the cells were subcloned by limiting dilution into 96-well plates so that a single antibody producing cell was in culture. As the culture became confluent, the cells were expanded to 1 ml, 3 ml, 5 ml, etc. and frozen

aliquots were stored in liquid nitrogen to preserve the cell stocks.

Using the foregoing procedures, antibodies specific for the antigens described above are prepared.

In accordance with the above procedure, mouse hybridomas producing human antibody against human IgE/λ were obtained.

In accordance with the above procedures, a chimeric nonhuman host, particularly a murine host, may be produced which can be immunized to produce human antibodies or analogs specific for an immunogen. In this manner, the problems associated with obtaining human monoclonal antibodies are avoided, because the transgenic host can be immunized with immunogens which could not be used with a human host. Furthermore, one can provide for booster injections and adjuvants which would not be permitted with a human host. The resulting B cells may then be used for immortalization for the continuous production of the desired antibody. The immortalized cells may be used for isolation of the genes encoding the immunoglobulin or analog and be subjected to further molecular modification by methods such as *in vitro* mutagenesis or other techniques to modify the properties of the antibodies. These modified genes may then be returned to the immortalized cells by transfection to provide for a continuous mammalian cellular source of the desired antibodies. The subject invention provides for a convenient source of human antibodies, where the human antibodies are produced in analogous manner to the production of antibodies in a human host. The animal host cells conveniently provide for the activation and rearrangement of human DNA in the host cells for production of human antibodies.

In accordance with the subject invention, human antibodies can be produced to human immunogens, e.g., proteins, by immunization of the subject host mammal with human immunogens. The resulting antisera will be specific for the human immunogen and may be harvested from the serum of the host. The immunized host B cells may be used for immortalization, e.g., myeloma cell fusion, transfection, etc. to provide immortal cells, e.g., hybridomas, to produce monoclonal

antibodies. The antibodies, antiserum and monoclonal antibodies will be glycosylated in accordance with the species of the cell producing the antibodies. Rare variable regions of the Ig locus may be recruited in producing the antibodies, so that antibodies having rare variable regions may be obtained.

All productions and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Claims

1. A method to produce an immunoglobulin having fully human variable region or an analog thereof, specific for a desired antigen, which method comprises:

administering said antigen or an immunogenic portion thereof to a nonhuman animal under conditions to stimulate an immune response, whereby said animal produces B cells that secrete immunoglobulin specific for said antigen; wherein said nonhuman animal is characterized by being substantially incapable of producing endogenous heavy and light immunoglobulin chain variable regions, but capable of producing human immunoglobulin variable regions; and

recovering said immunoglobulin or analog.

2. The method of claim 1 wherein said recovering step comprises recovering polyclonal immunoglobulin or analog from said animal.

3. The method of claim 1 wherein said recovering step comprises immortalizing B cells from said animal immunized with said antigen, screening the resulting immortalized cells for the secretion of said immunoglobulin specific for said antigen, and

1) recovering immunoglobulin secreted by said immortalized B cells, or

2) recovering the genes encoding at least the variable region of said immunoglobulin from the immortalized B cells, and optionally modifying said genes;

expressing said genes or modified forms thereof to produce immunoglobulin or analog; and

recovering said immunoglobulin or analog.

4. The method of claim 1 wherein said recovering step comprises

recovering genes encoding at least the variable region of immunoglobulins from the primary B cells of the animal immunized with said antigen;

generating a library of said genes expressing the variable regions;

screening the library for a variable region with desired affinity for the antigen;

recovering the genes encoding said variable regions and optionally modifying said genes;

expressing said recovered genes to produce an immunoglobulin or analog containing said variable region and recovering said immunoglobulin or analog.

5. The method of claim 1 wherein said immunoglobulin is fully human.

6. A recombinant DNA molecule comprising a nucleotide sequence encoding the immunoglobulin or analog produced by the method of claim 1.

7. A recombinant DNA molecule comprising an encoding nucleotide sequence corresponding to a gene prepared by a method comprising

administering a desired antigen or an immunogenic portion thereof to a nonhuman animal under conditions to stimulate an immune response, whereby said animal produces B cells that secrete immunoglobulin specific for said antigen; wherein said nonhuman animal is characterized by being substantially incapable of producing endogenous heavy and light immunoglobulin chain variable regions, but capable of producing human immunoglobulin variable regions;

immortalizing B cells from said animal immunized with said antigen, screening the resulting immortalized cells for the secretion of said immunoglobulin specific for said antigen, and

recovering the genes encoding at least the variable region of said immunoglobulin from the immortalized B cells, and optionally modifying said genes.

8. A recombinant DNA molecule comprising an encoding nucleotide sequence corresponding to a gene prepared by a method comprising

administering a desired antigen or an immunogenic portion thereof to a nonhuman animal under conditions to stimulate an immune response, whereby said animal produces B cells that secrete immunoglobulin specific for said antigen; wherein said nonhuman animal is characterized by being substantially incapable of producing endogenous heavy and light immunoglobulin chain variable regions, but capable of producing human immunoglobulin variable regions;

recovering genes encoding at least the variable region of immunoglobulins from the primary B cells of the animal immunized with said antigen;

generating a library of said genes expressing the variable regions;

screening the library for a variable region with desired affinity for the antigen; and

recovering the genes encoding said variable regions and optionally modifying said genes.

9. The DNA molecule of claim 6, 7 or 8 wherein said encoding nucleotide sequence is operably linked to control sequences capable of effecting its expression.

10. A cell or cell line modified to contain the DNA molecule of claim 9.

11. A method to produce an immunoglobulin with fully human variable region or an analog thereof which method comprises culturing the cells of claim 10 under conditions whereby said encoding nucleotide sequence is expressed to produce said immunoglobulin or analog; and

recovering said immunoglobulin or analog.

12. An immortalized B cell which secretes an immunoglobulin with a fully human variable region to a desired antigen prepared by a method which comprises

administering said antigen or an immunogenic portion thereof to a nonhuman animal under conditions to stimulate an immune response, whereby said animal produces B cells that

secrete immunoglobulin specific for said antigen; wherein said nonhuman animal is characterized by being substantially incapable of producing endogenous heavy and light immunoglobulin chain variable regions, but capable of producing human immunoglobulin variable regions;

immortalizing B cells from said animal immunized with said antigen, screening the resulting immortalized cells for the secretion of said immunoglobulin specific for said antigen; and

recovering said immortalized B cell.

13. A method to produce an immunoglobulin or analog which comprises culturing the recovered cells of claim 12 and recovering said immunoglobulin or analog.

14. An immunoglobulin with fully human variable region or analog thereof produced by the method of claim 1.

15. The immunoglobulin or analog of claim 14 which is fully human.

16. The immunoglobulin or analog of claim 14 which is an agonist or a catalyst or wherein the immunoglobulin is chimeric.

17. The immunoglobulin or analog of claim 14 wherein the desired antigen is selected from the group consisting of transition state mimics; leukocyte markers; histocompatibility antigens; adhesion molecules; interleukins; interleukin receptors; chemokines; growth factors; growth factor receptors; interferon receptors; Igs and their receptors; tumor antigens; allergens; viral proteins; toxins; blood factors; enzymes; and the miscellaneous antigens ganglioside GD3, ganglioside GM2, LMP1, LMP2, eosinophil major basic protein, eosinophil cationic protein, pANCA, Amadori protein, Type IV collagen, glycated lipids, γ-interferon, A7, P-glycoprotein, Fas (AFO-1) and oxidized-LDL.

18. The immunoglobulin or analog of claim 17 wherein the leukocyte marker is selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD11a,b,c, CD13, CD14, CD18, CD19, CD20, CD22, CD23, CD27 and its ligand, CD28 and its ligands B7.1, B7.2, B7.3, CD29 and its ligand, CD30 and its ligand, CD40 and its ligand gp39, CD44, CD45 and isoforms, CDw52 (Campath antigen), CD56, CD58, CD69, CD72, CTLA-4, LFA-1 and TCR;

the histocompatibility antigen is selected from the group consisting of MHC class I or II, the Lewis Y antigens, SLex, SLey, SLea, and SLeb;

the adhesion molecule is selected from the group consisting of VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, LFA-1, L-selectin, P-selectin, and E-selectin and their counterreceptors VCAM-1, ICAM-1, ICAM-2, LFA-3; Mac-1 and p150,95;

the interleukin is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15;

the interleukin receptor is selected from the group consisting of IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-8R, IL-9R, IL-10R, IL-11R, IL-12R, IL-13R, IL-14R, and IL-15R;

the chemokine is selected from the group consisting of PF4, RANTES, MIP1α, MCP1, NAP-2, Groα, Groβ, and IL-8;

the growth factor is selected from the group consisting of TNFalpha, TGFbeta, TSH, VEGF/VPF, PTHrP, EGF family, FGF, PDGF family, endothelin, and gastrin releasing peptide (GRP);

the growth factor receptor is selected from the group consisting of TNFalphaR, RGFbetaR, TSHR, VEGFR/VPFR, FGFR, EGFR, PTHrPR, PDGFR family, EPO-R, GCSF-R and other hematopoietic receptors;

the interferon receptor is selected from the group consisting of IFNαR, IFNβR, and IFNγR;

the Ig and its receptor is selected from the group consisting of IgE, FceRI, and FCeRII;

the tumor antigen is selected from the group consisting of her2-neu, mucin, CEA and endosialin;

the allergen is selected from the group consisting of house dust mite antigen, lol p1 (grass) antigens, and urushiol;

the viral protein is selected from the group consisting of CMV glycoproteins B, H, and gCIII, HIV-1 envelope glycoproteins, RSV envelope glycoproteins, HSV envelope glycoproteins, EBV envelope glycoproteins, VZV envelope glycoproteins, HPV envelope glycoproteins, Hepatitis family surface antigens;

the toxin is selected from the group consisting of pseudomonas endotoxin and osteopontin/uropontin, snake venom, and bee venom;

the blood factor is selected from the group consisting of complement C3b, complement C5a, complement C5b-9, Rh factor, fibrinogen, fibrin, and myelin associated growth inhibitor; and

the enzyme is selected from the group consisting of cholesterol ester transfer protein, membrane bound matrix metalloproteases, and glutamic acid decarboxylase (GAD).

19. The immunoglobulin or analog of claim 14 wherein said desired antigen is selected from the group consisting of human IL-6, human IL-8, human TNFα, human CD4, human L-selectin, human gp39, human IgE and tetanus toxin C(TTC).

20. A recombinant DNA molecule comprising a nucleotide sequence that encodes the immunoglobulin or analog of any of claims 15-19.

21. The DNA molecule of claim 20 wherein said encoding nucleotide sequence is operably linked to control sequences capable of effecting its expression.

22. A cell or cell line modified to contain the DNA molecule of claim 21.

23. A method to produce an immunoglobulin or analog specific for a desired antigen which method comprises culturing

the cell or cell line of claim 22 under conditions wherein said nucleotide sequence is expressed to produce said immunoglobulin or analog; and

recovering the immunoglobulin or analog.

24. An antibody containing a fully human variable region or analog thereof which is specifically immunoreactive with an antigen selected from the group consisting of transition state mimics; leukocyte markers; histocompatibility antigens; adhesion molecules; interleukins; interleukin receptors; chemokines; growth factors; growth factor receptors; interferon receptors; Igs and their receptors; tumor antigens; allergens; viral proteins; toxins; blood factors; enzymes; and the miscellaneous antigens ganglioside GD3, ganglioside GM2, LMP1, LMP2, eosinophil major basic protein, eosinophil cationic protein, pANCA, Amadori protein, Type IV collagen, glycated lipids, γ-interferon, A7, P-glycoprotein, Fas (AFO-1) and oxidized-LDL.

25. The antibody or analog of claim 24 wherein the leukocyte marker is selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD11a,b,c, CD13, CD14, CD18, CD19, CD20, CD22, CD23, CD27 and its ligand, CD28 and its ligands B7.1, B7.2, B7.3, CD29 and its ligand, CD30 and its ligand, CD40 and its ligand gp39, CD44, CD45 and isoforms, CDw52 (Campath antigen), CD56, CD58, CD69, CD72, CTLA-4, LFA-1 and TCR;

the histocompatibility antigen is selected from the group consisting of MHC class I or II, the Lewis Y antigens, SLex, SLey, SLea, and SLeb;

the adhesion molecule is selected from the group consisting of VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, LFA-1, L-selectin, P-selectin, and E-selectin and their counterreceptors VCAM-1, ICAM-1, ICAM-2, LFA-3; Mac-1 and p150,95;

the interleukin is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15;

the interleukin receptor is selected from the group consisting of IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-8R, IL-9R, IL-10R, IL-11R, IL-12R, IL-13R, IL-14R, and IL-15R;

the chemokine is selected from the group consisting of PF4, RANTES, MIP1α, MCP1, NAP-2, Groα, Groβ, and IL-8;

the growth factor is selected from the group consisting of TNFalpha, TGFbeta, TSH, VEGF/VPF, PTHrP, EGF family, FGF, PDGF family, endothelin, and gastrin releasing peptide (GRP);

the growth factor receptor is selected from the group consisting of TNFalphaR, RGFbetaR, TSHR, VEGFR/VPFR, FGFR, EGFR, PTHrPR, PDGFR family, EPO-R, GCSF-R and other hematopoietic receptors;

the interferon receptor is selected from the group consisting of IFNαR, IFNβR, and IFNγR;

the Ig and its receptor is selected from the group consisting of IgE, FceRI, and FCeRII;

the tumor antigen is selected from the group consisting of her2-neu, mucin, CEA and endosialin;

the allergen is selected from the group consisting of house dust mite antigen, lol p1 (grass) antigens, and urushiol;

the viral protein is selected from the group consisting of CMV glycoproteins B, H, and gCIII, HIV-1 envelope glycoproteins, RSV envelope glycoproteins, HSV envelope glycoproteins, EBV envelope glycoproteins, VZV envelope glycoproteins, HPV envelope glycoproteins, Hepatitis family surface antigens;

the toxin is selected from the group consisting of pseudomonas endotoxin and osteopontin/uropontin, snake venom, and bee venom;

the blood factor is selected from the group consisting of complement C3b, complement C5a, complement C5b-9, Rh factor, fibrinogen, fibrin, and myelin associated growth inhibitor; and

the enzyme is selected from the group consisting of cholesterol ester transfer protein, membrane bound matrix metalloproteases, and glutamic acid decarboxylase (GAD).

26. The antibody or analog of claim 24 wherein the desired antigen is selected from the group consisting of human IL-6, human IL-8, human TNFα, human CD4, human L-selectin, human gp39, human IgE and tetanus toxin C(TTC).

27. The antibody or analog of claim 19 or 26 wherein the desired antigen is human IL-6.

28. The antibody or analog of claim 19 or 26 wherein the desired antigen is human IL-8.

29. The antibody or analog of claim 19 or 26 wherein the desired antigen is human TNFα.

30. The antibody or analog of claim 19 or 26 wherein the desired antigen is human CD4.

31. The antibody or analog of claim 19 or 26 wherein the desired antigen is human L-selectin.

32. The antibody or analog of claim 19 or 26 wherein the desired antigen is human gp39.

33. The antibody or analog of claim 19 or 26 wherein the desired antigen is tetanus toxin C(TTC).

34. The antibody or analog of claim 19 or 26 wherein the desired antigen is human IgE.

35. The analog of claim 19 or 26 which is a single chain F_V.

36. The antibody or analog of claim 24 which is fully human.

37. The antibody or analog of claim 24 which is an agonist or is a catalyst or wherein the immunoglobulin is chimeric.

38. A recombinant DNA molecule encoding the antibody or analog of any of claims 26-37.

39. A recombinant DNA molecule which comprises an expression system for the production of the antibody or analog of any of claims 26-37 which expression system comprises a nucleotide sequence encoding said antibody or analog operably linked to control sequences capable of effecting its expression.

40. A recombinant host cell which is modified to contain the DNA molecule of claim 39.

41. A method to produce an antibody or analog which method comprises culturing the cells of claim 40 under conditions wherein said coding sequence is expressed; and recovering the antibody or analog produced.

42. Use of the antibody or analog of claim 36 for *in vivo* prophylaxis, therapy or diagnosis in humans.

43. Use of the antibody or analog of claim 27, 29, 30, 31 or 32 for treating an autoimmune disease in a mammal.

44. The use of claim 43 wherein the autoimmune disease is systemic lupus erythrematosus, rheumatoid arthritis, psoriasis, Sjogren's syndrome, scleroderma, mixed connective tissue disease, dermatomyositis, polymyositis, Reiter's syndrome, Behcet's disease, Type I diabetes, Hashimoto's thyroiditis, Graves' disease, multiple sclerosis, myasthenia gravis, or pemphigus.

45. Use of the antibody of claim 32 for preventing graft versus host disease, for preventing rejection of an organ transplant, or for treating glomerular nephritis in a mammal.

46. Use of the antibody of claim 31 for treating reperfusion ischemia in a mammal.

47. Use of the antibody of claim 27 for treating cachexia, septic shock, myeloma, renal cell carcinoma, osteoporosis, or Paget disease in a mammal.

48. Use of the antibody of claim 29 for treating septic shock, cachexia, osteoporosis, or systemic sclerosis in a mammal.

49. Use of the antibody of claim 28 for preventing tumor metastasis, and for treating asthma, rheumatoid arthritis, glomerulonephritis, reperfusion injury, adult respiratory distress syndrome, or systemic sclerosis in a mammal.

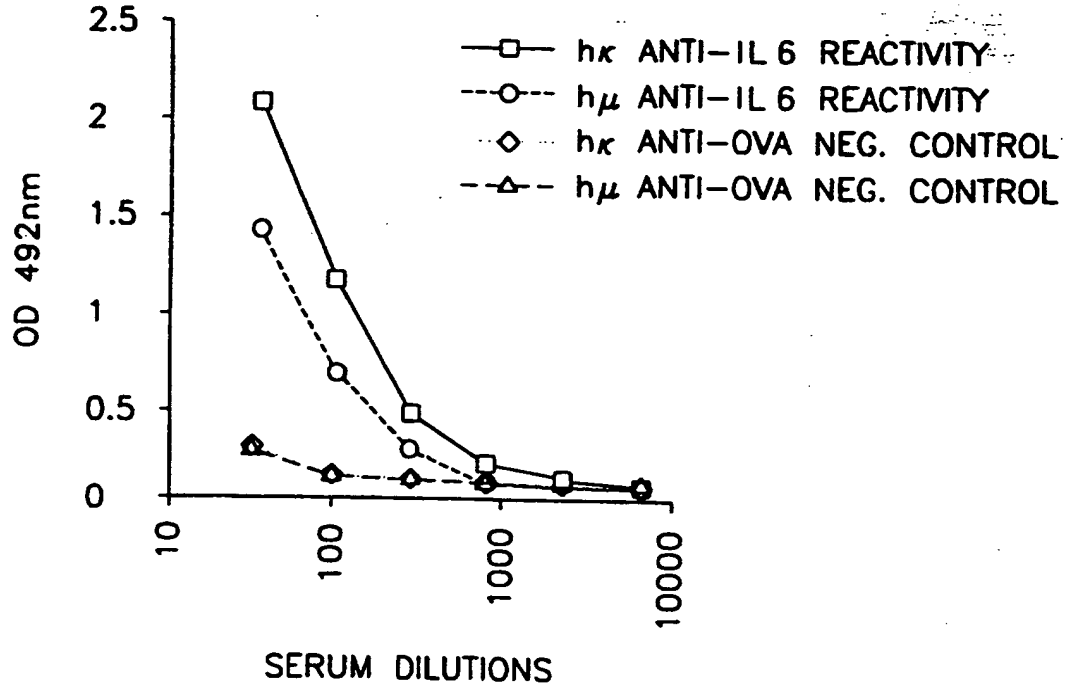


FIG. 1

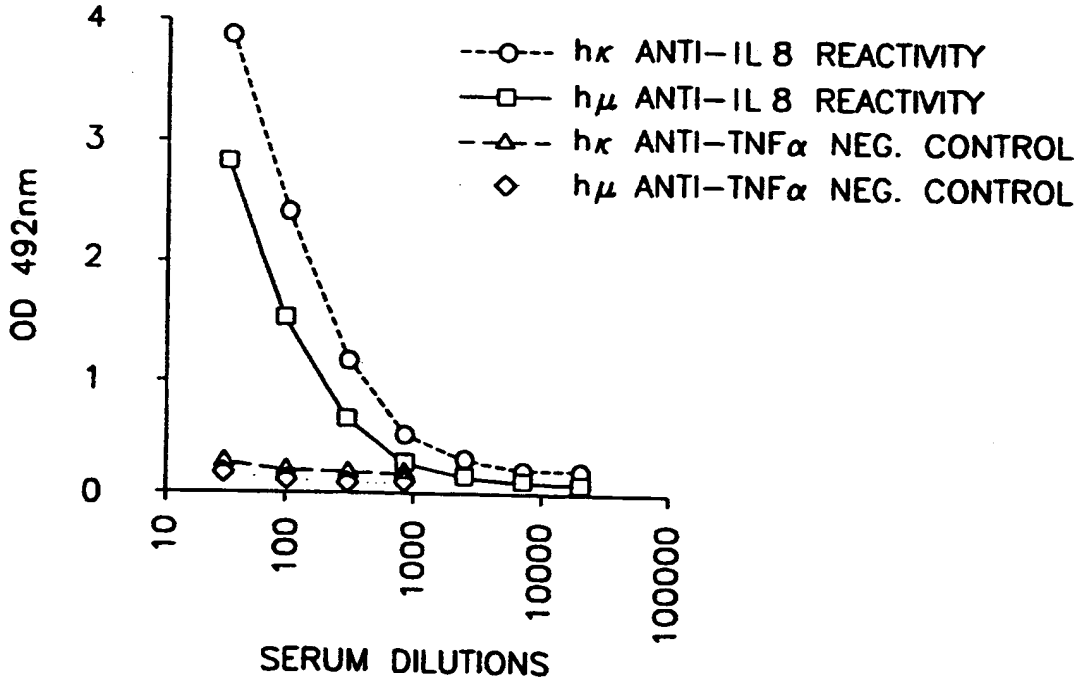


FIG. 2

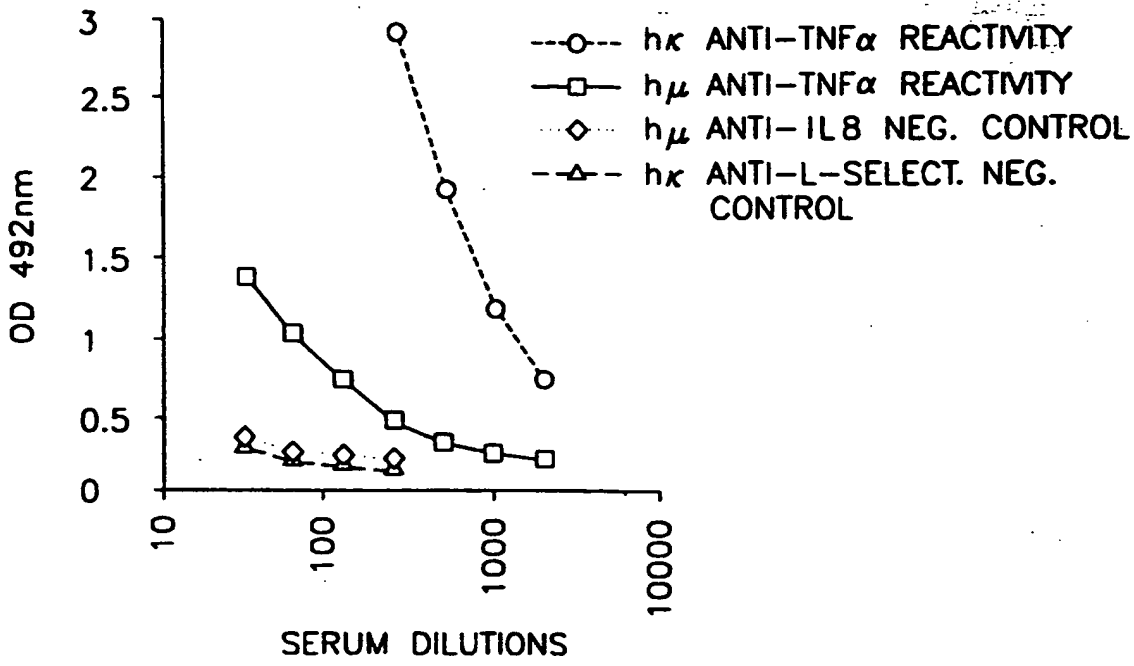


FIG. 3

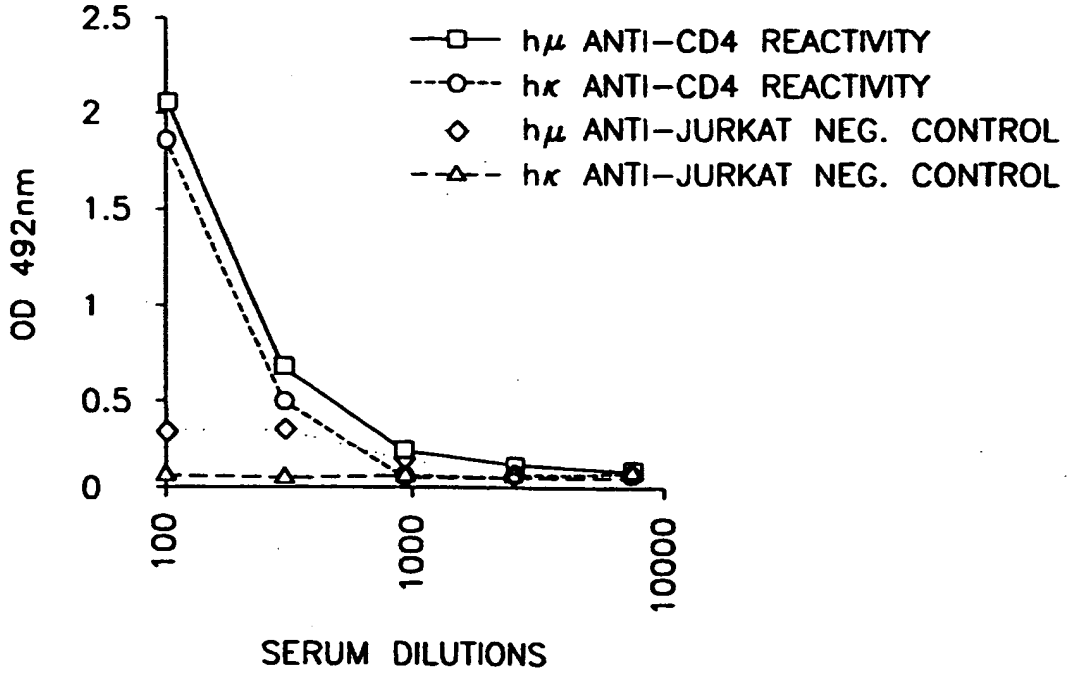


FIG. 4

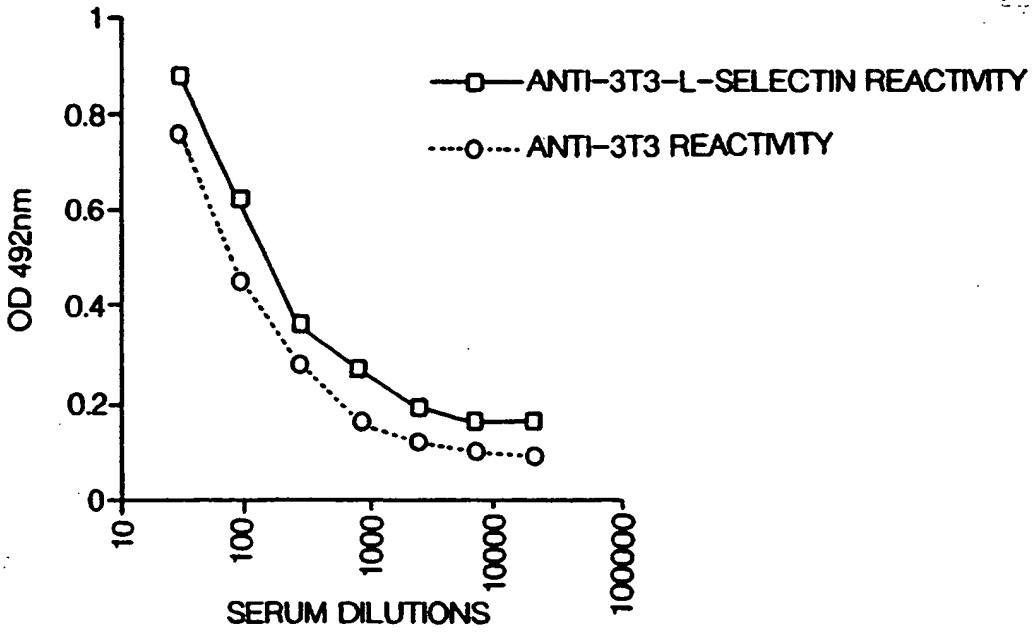


FIG. 5

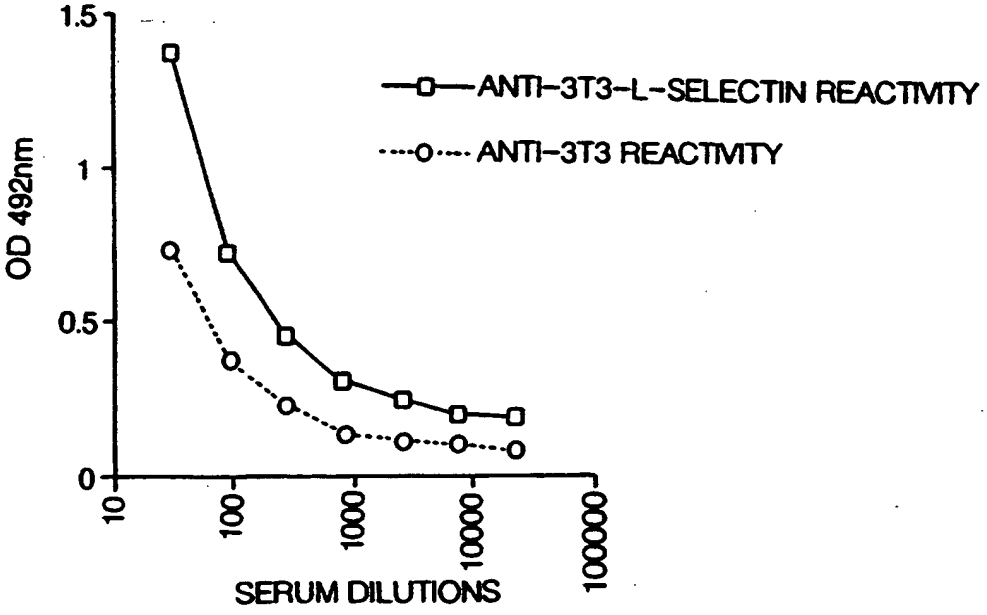


FIG. 6

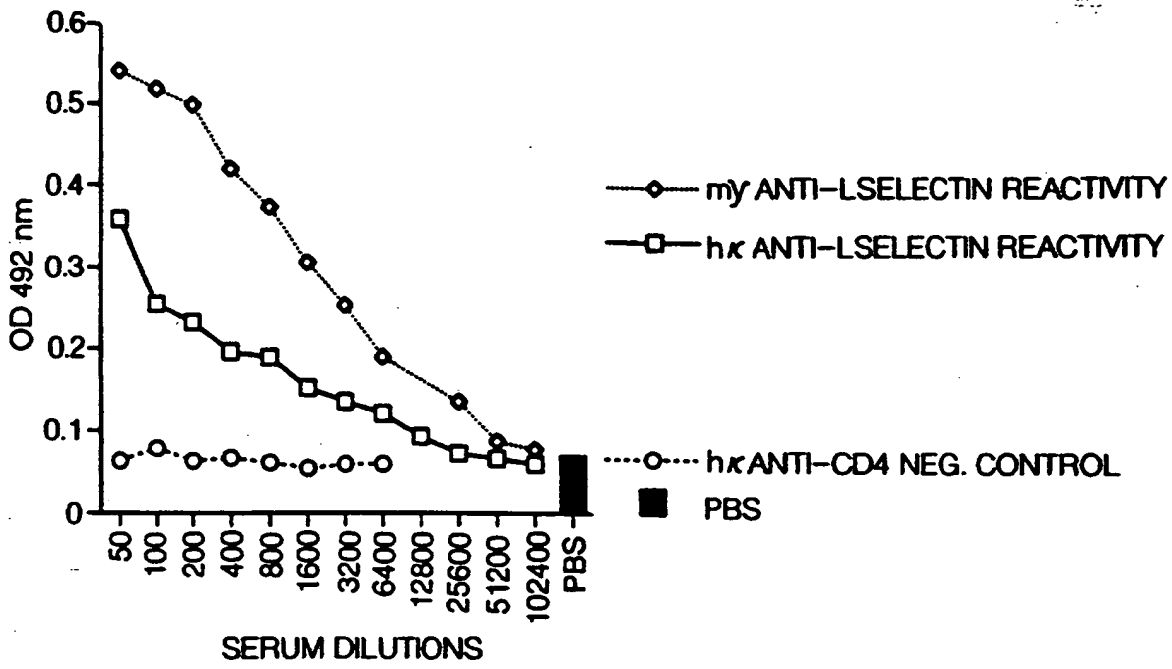


FIG. 7

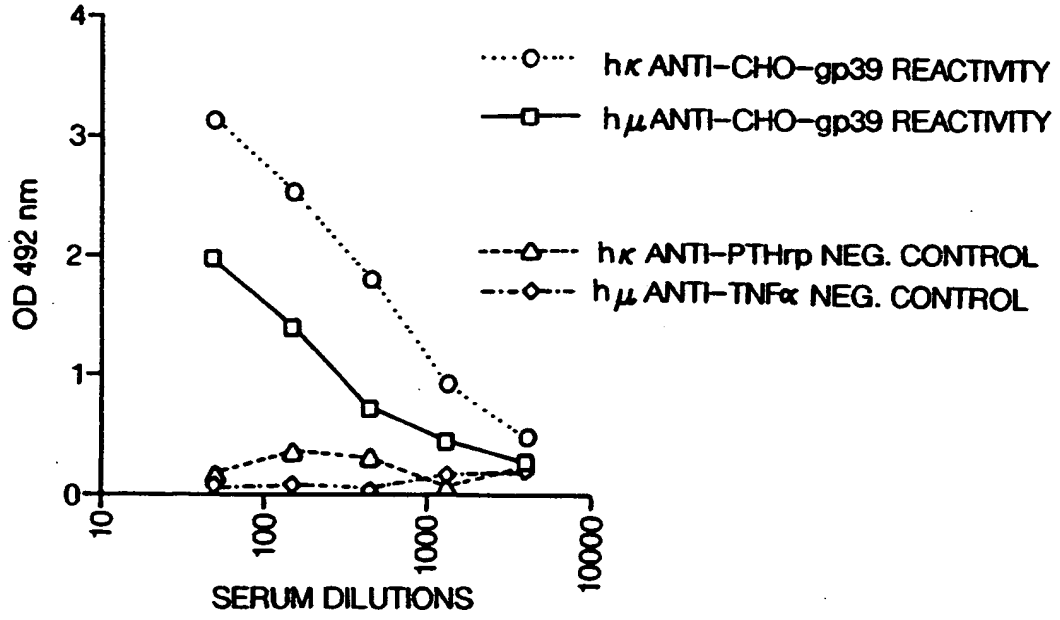


FIG. 11

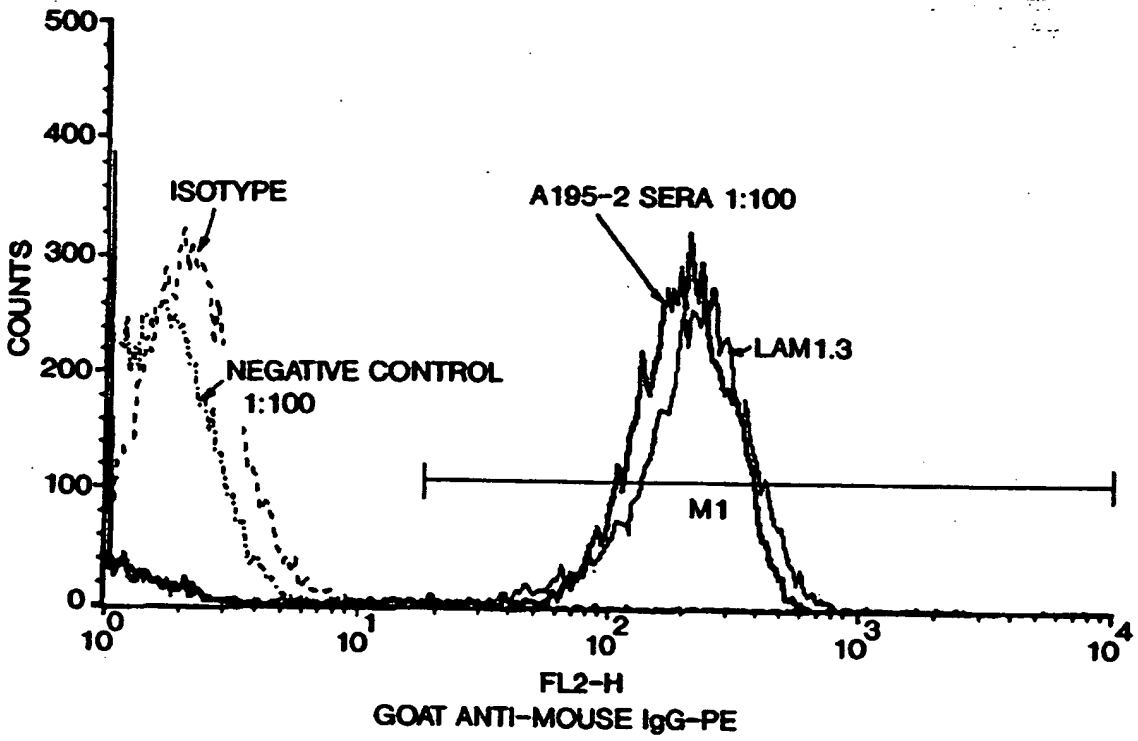


FIG. 8

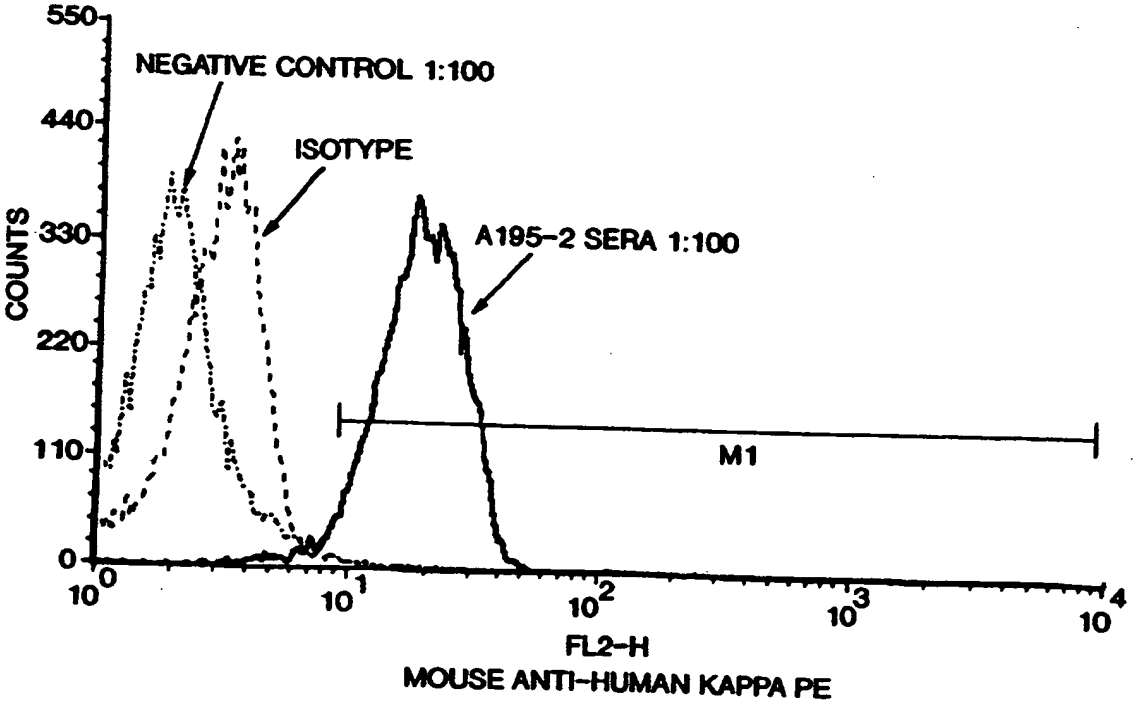
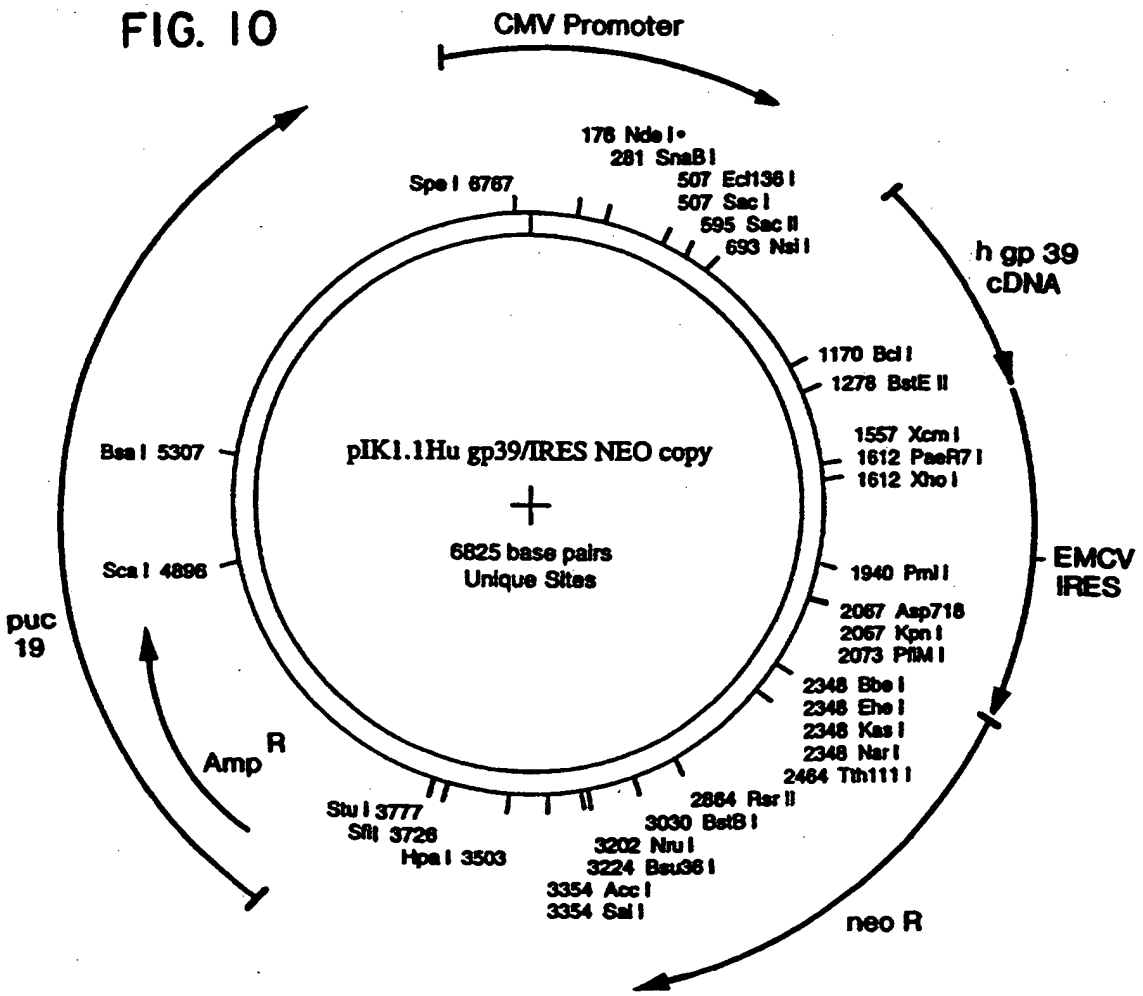


FIG. 9

FIG. 10



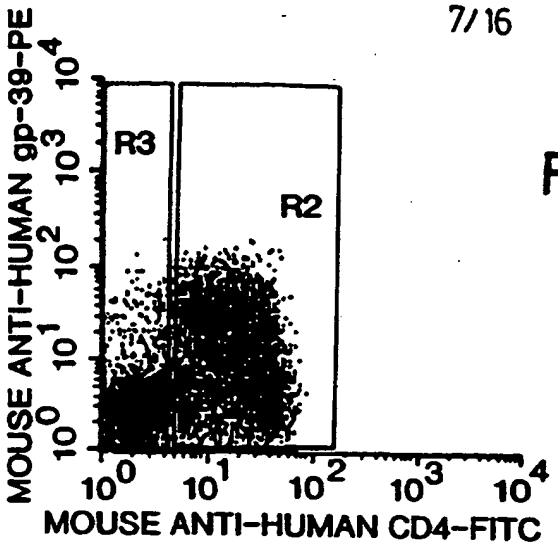


FIG. 12A

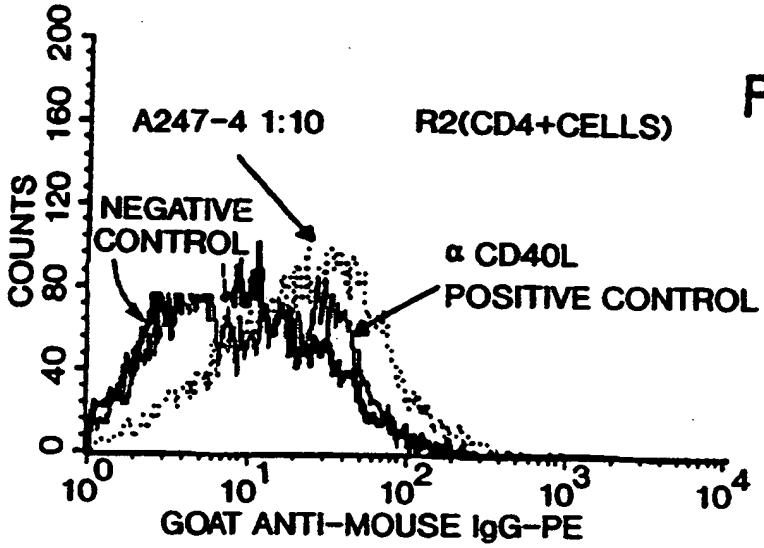


FIG. 12B

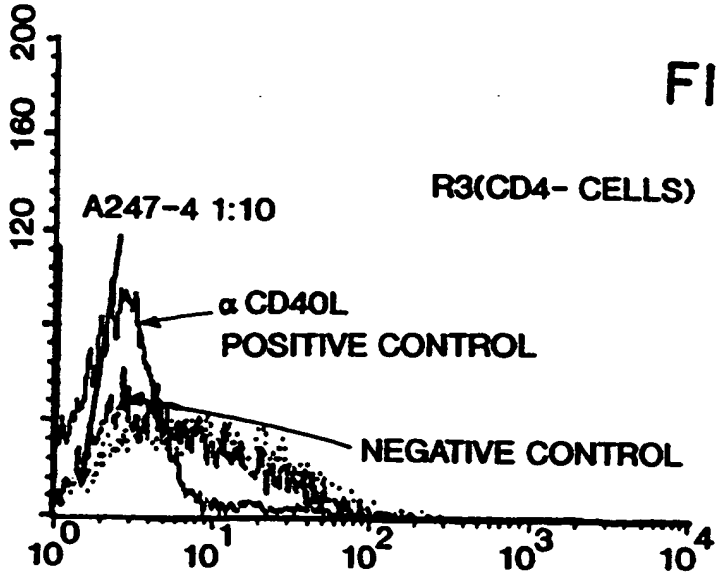


FIG. 12C

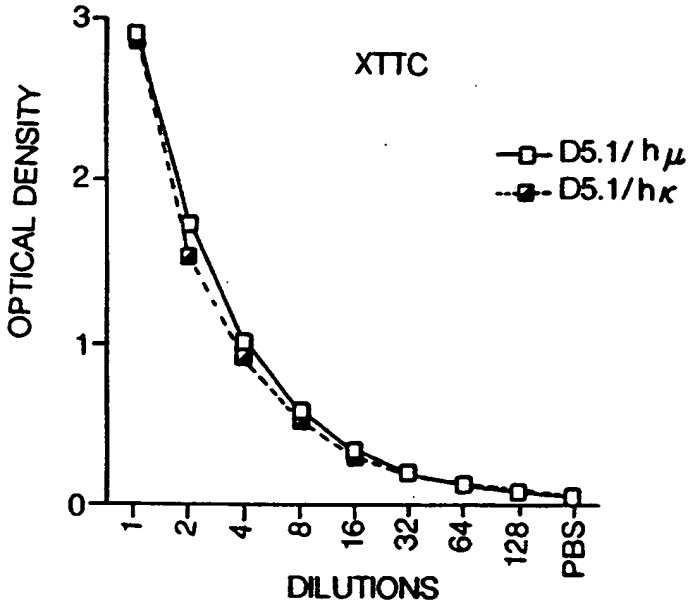


FIG. 13

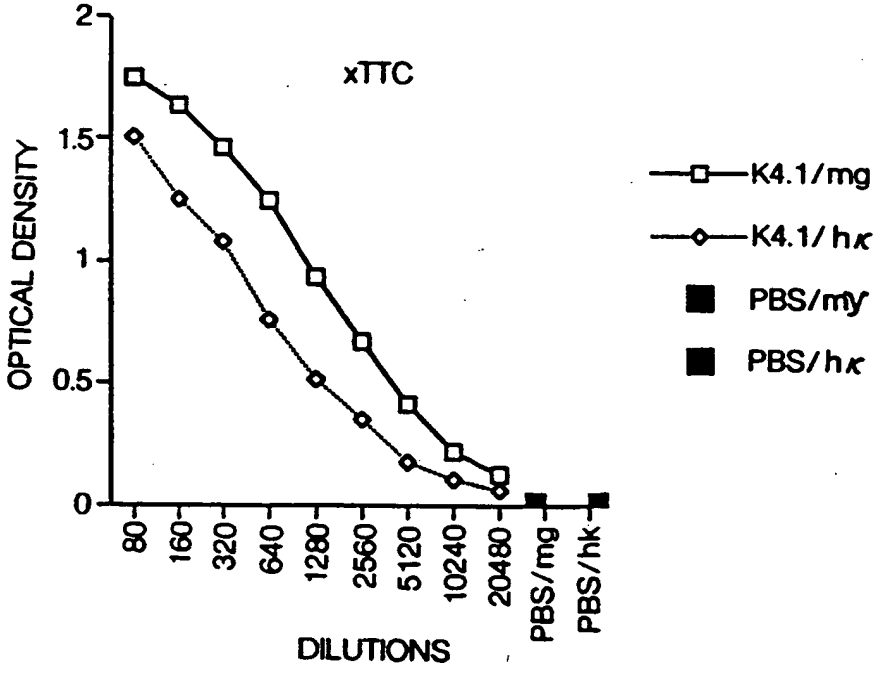


FIG. 14

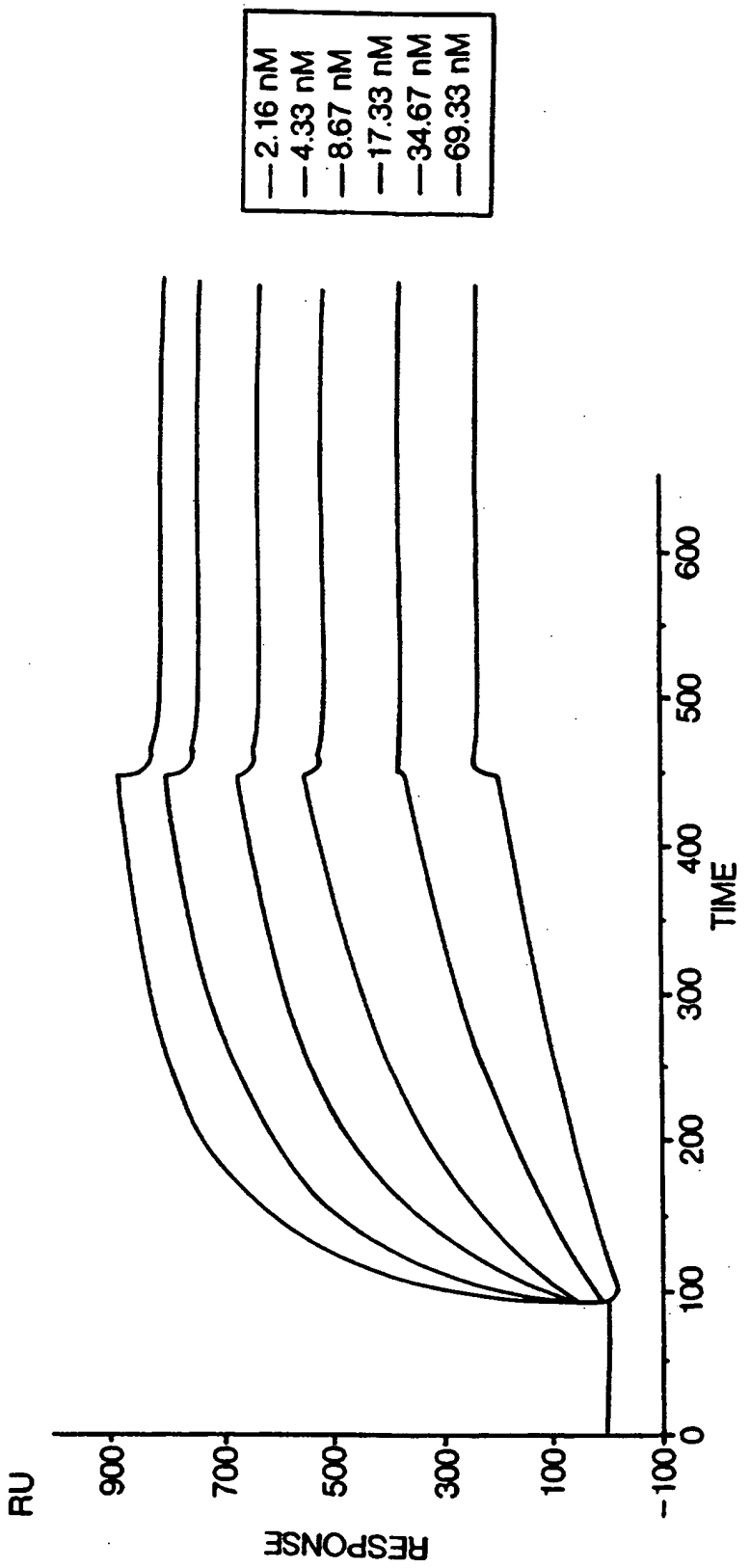


FIG. 15

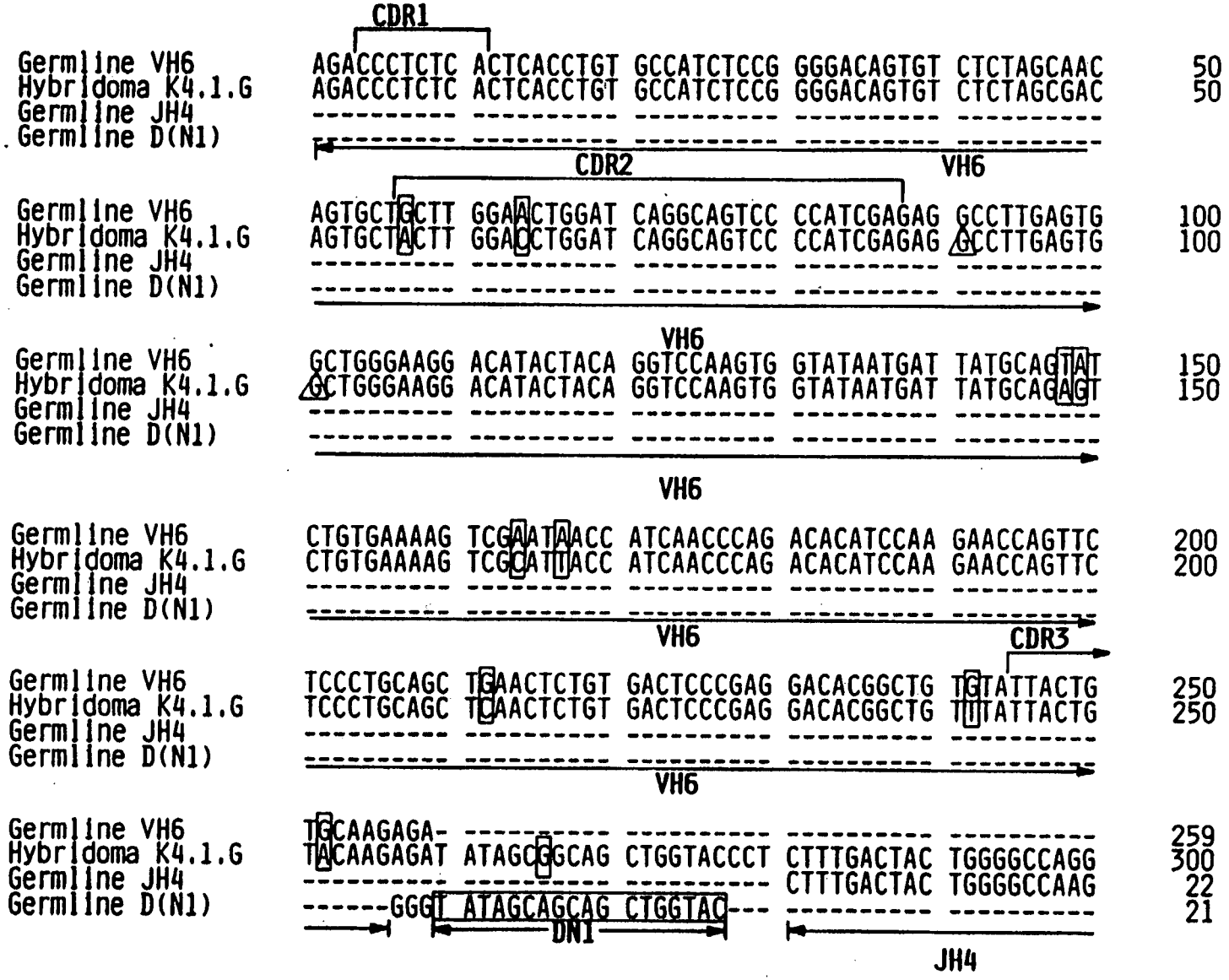


FIG. 16A

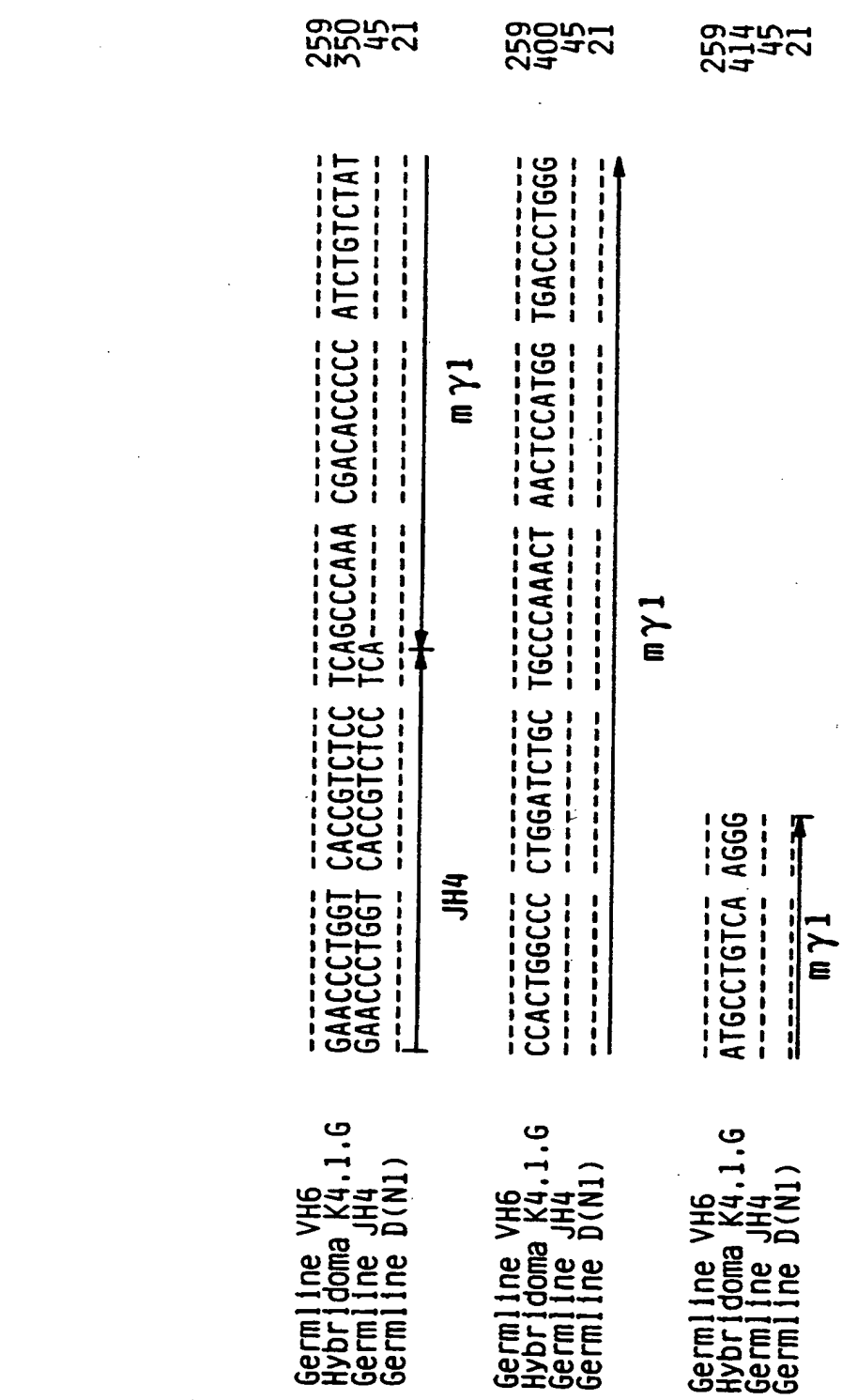


FIG. 16B

FIG. 17

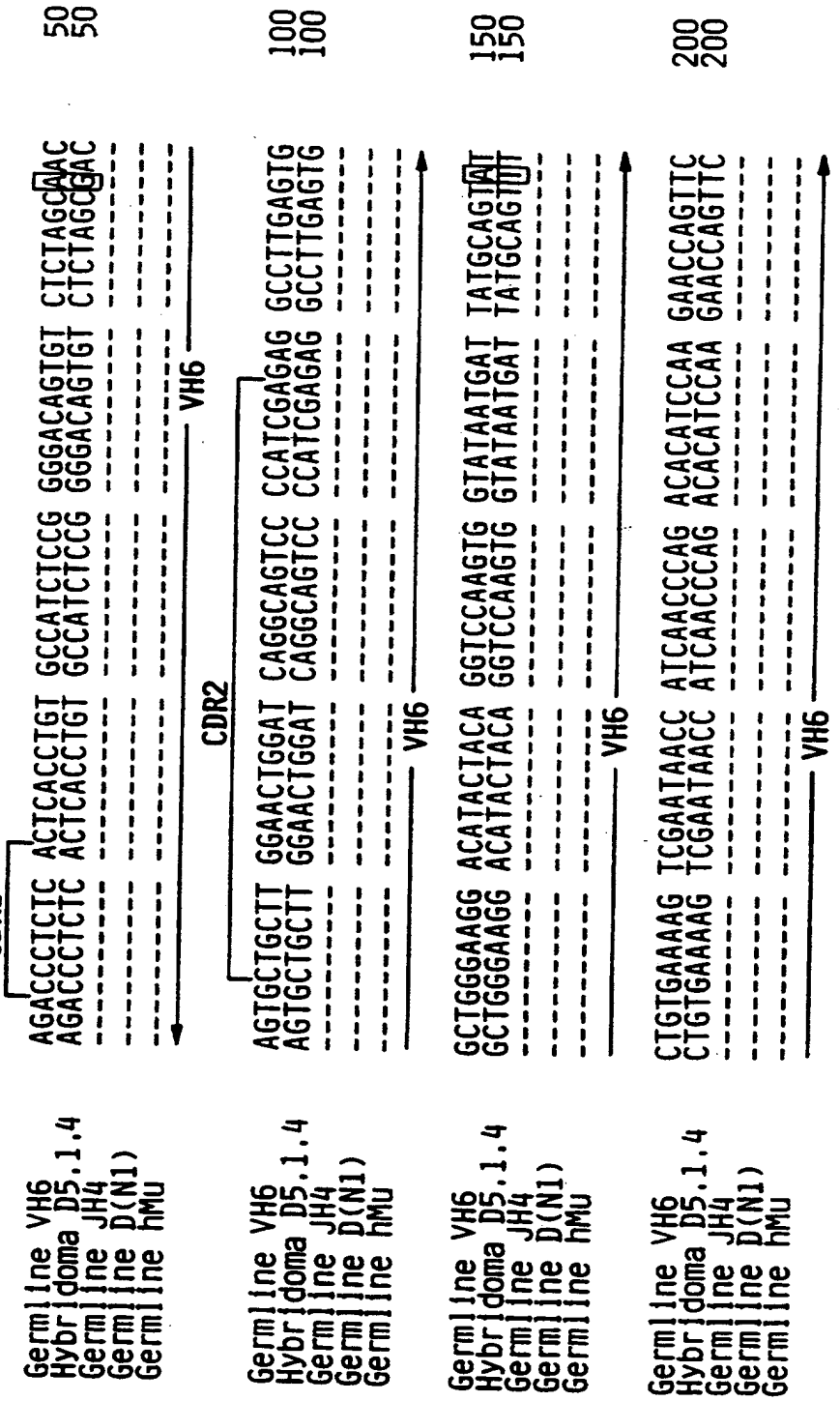


FIG. 18A

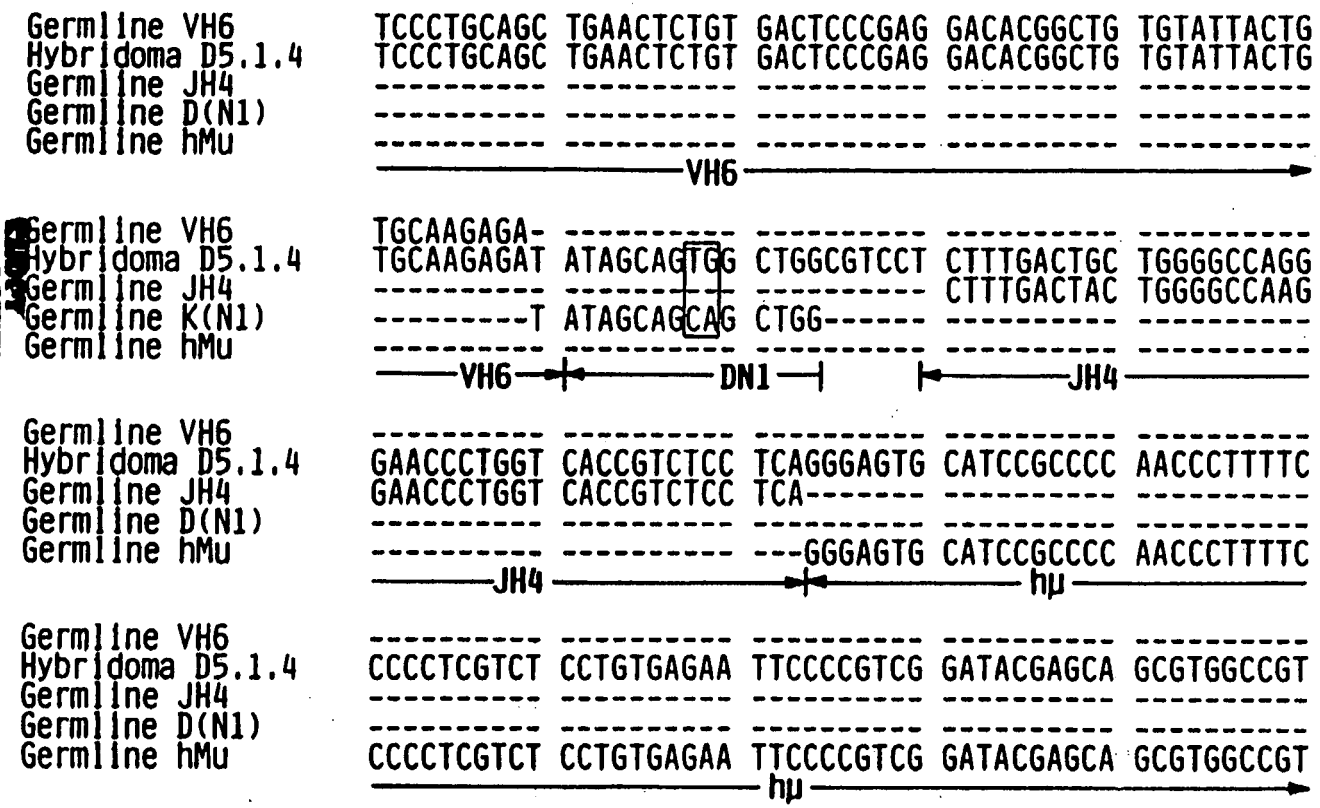


FIG. 18B

PCT/US95/05500

FIG. 19A

```
Germline B3         TGCAGGCTGA AGATGTGGCA GTTTATTACT GTCAGCAATA TTATAGTACT
Hybridoma D5.1.4    TGCAGGCTGA AGATGTGGCA GTTTATTACT GTCAGCAATA TTATACTATT
Germline JK3        ---------- ---------- ---------- ---------- ----------
Germline CK         ---------- ---------- ---------- ---------- ----------
                    ---------------------------B3--------------------------->

Germline B3         CC-------- ---------- ---------- ---------- ----------
Hybridoma D5.1.4    CCATTCAATT TCGGCCCTGG GACCAGAGTG GATATCAAAC GAACTGTGGC
Germline JK3        --ATTCACTT TCGGCCCTGG GACCAAAGTG GATATCAAAC ----------
Germline CK         ---------- ---------- ---------- ---------- GAACTGTGGC
                    -->|<-------------------JK3------------------>|<---CK---

Germline B3         ---------- ---------- ---------- ---------- ----------
Hybridoma D5.1.4    TGCACCATCT GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG
Germline JK3        ---------- ---------- ---------- ---------- ----------
Germline CK         TGCACCATCT GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG
                    ---------------------------CK--------------------------->

Germline B3         ---------- ---------- ---------- ---------- ----------
Hybridoma D5.1.4    GAACTGCCTC TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC
Germline JK3        ---------- ---------- ---------- ---------- ----------
Germline CK         GAACTGCCTC TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC
                    ---------------------------CK--------------------------->

Germline B3         ---------- ---------- ---------- ---------- ---------
Hybridoma D5.1.4    AAAGTACAGT GGAAGGTGGA TAACGCCCTC CAATCGGGTT GGGGAAAAA
Germline JK3        ---------- ---------- ---------- ---------- ---------
Germline CK         AAAGTACAGT GGAAGGTGGA TAACGCCCTC CAATCGGGT- ---------
                    ---------------------------CK-------------------------->
```

FIG. 19B

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05500

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/00
US CL :435/172.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog
search terms: transgenic, mice, human chimeric immunoglobulin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 244, issued 16 June 1989, (Capecchi), "Altering the Genome by Homologous Recombination", pages 1288-1292, see entire article.	1-19, 27-35
Y	Journal of Immunological Methods, volume 100, issued 1987, (James et al.), "Human monoclonal antibody production: current status and future prospects", pages 5-40, see entire reference.	24-37, 42
Y	WO, A, 91/00906 (GENETICS INSTITUTE, INC.) 24 January 1991, see entire document.	1-19, 24-37, 42

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
27 JULY 1995

Date of mailing of the international search report
07 SEP 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer
Suzanne Ziska, Ph.D.

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	European Journal of Immunology, Volume 21, issued 1991, (Bruggemann et al.), "Human antibody production in transgenic mice: expression from 100kb of the human IgH locus", pages 1323-1326, see entire article.	1-19, 24-37, 42
Y	Gene, Volume 87, issued 1990, (Lenz et al.), "Expression of heterobispecific antibodies by genes transferred into producer hybridoma cells", pages 213-218, see entire reference.	5-10
Y	Nature, volume 336, issued 24 November 1988, (Mansour et al.), "Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes", pages 348-352, see entire reference.	1-19, 24-37, 42
Y	EP, A, 0,315,062 (BRISTOL-MYERS COMPANY) 10 May 1989, see entire document.	1-19, 24-37, 42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05500

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 20-23, 38-41
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-42

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05500

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-42, drawn to a first method of producing an immunoglobulin having a fully human variable region, the immunoglobulin produced by the method, and the first method of using the immunoglobulin for therapy or diagnosis in humans.

Group II, claim(s) 43 and 44, drawn to a second method of using the immunoglobulins, for treatment of an autoimmune disease in a mammal.

Group III, claim 45, drawn to a third method of using the immunoglobulins, for treatment of organ rejection.

Group IV, claim 46, drawn to drawn to a fourth method of using the immunoglobulins, for treatment of reperfusion ischemia in a mammal.

Group V, claims 47 and 48, drawn to a fifth method of using the immunoglobulins, for treatment of cachexia.

Group VI, claim 49, drawn to a sixth method of using the immunoglobulins, for treatment of tumor metastasis.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

each antibody needed for each treatment is a different antibody and each different antibody is the special technical feature which is different for each method. Each method requires a different antibody and therefore each group requires the use of an antibody having a different technical feature. Each method uses different antibodies which have different technical features because each antibody is different. Each antibody is different because each antibody recognizes a different antigen having a different primary, secondary and tertiary structure. The structure of each antibody variable region, the region involved in antigen recognition, is encoded by the DNA and therefore immunization with each different antigen induces a different arrangement of genomic immunoglobulin DNA, resulting in a DNA sequence unique for the antibody recognizing a specific antigen.
The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.